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(54) Title: METHOD AND DEVICE FOR MICROINJECTION OF MACROMOLECULES INTO NON-ADHERENT CELLS			
(57) Abstract <p>The present invention is directed to a method of incorporating foreign material into cells that exist in a non-adherent state. In some embodiments, the method employs a microinjection needle together with an adhesive molecule or apparatus capable of immobilizing cells that exist in a non-adherent state onto a substrate or other surface capable of supporting cell attachment to permit introduction of a desired material, such as nucleic acid, into the cell. The non-adherent cells can be genetically altered by the microinjection of nucleic acid sequences (DNA) of sufficient size to permit direct introduction of said nucleic acid into the chromosomal DNA of the cell, without the use of a retrovirus, adenovirus, or other carrier molecule. The invention also thus provides a method of gene therapy insofar as cells modified according to the defined method, particularly undifferentiated cells such as stem cells, may be created to include a gene or fragment thereof sufficient to replace a deficiency or correct an abnormality or deficiency in the cell, and used in a patient by injection of the cells. The corrected nucleic acid then gives rise to genetically corrected differentiated cell species, and hence an approach useful in treating a number of physiological disorders.</p>			

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## METHOD AND DEVICE FOR MICROINJECTION OF MACROMOLECULES INTO NON-ADHERENT CELLS

This application claims the benefit of U.S. Provisional Application No.: 60/033,820, filed December 20, 1996.

### TECHNICAL FIELD

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The present invention relates to the field of molecular transfer of materials into living cells, and to improved methods for accomplishing same, particularly in the incorporation of molecular materials into cells that can be made to exist in an adherent state in vitro. The invention further relates to the field of gene therapy, as a technique for introducing genetic material into a cell is provided. The invention also relates to the field of mechanical apparatus for micro-injecting molecular materials into living cells, devices for introducing materials into a cell with improved cell viability and retention/expression of incorporated materials therein, also being provided in the present disclosure.

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### BACKGROUND ART

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Microinjection of macromolecules (e.g., antibodies, mRNA, DNA) into living cells has proven to be a powerful approach for studying the biology of cells at the molecular level. Manual microinjection methods have been developed independently by Diacumakos and Graessmann [Diacumakos, E. (1973); Graessmann, A. (1970)], two pioneers of microinjection technology. The methods employ a micromanipulator that is used in directing a glass microinjection needle into a living cell. The microinjection needle is connected to a syringe assembly that forces the sample out of the needle. The flow of the sample solution into the cell is typically visually monitored employing a phase-contrast microscope. In this manner, a change in phase contrast indicates that the sample has been injected into the cell. These methods require extensive training of the personnel who perform the injections; this training period lasts for months before acceptable consistent injections can be accomplished. These manual microinjection approaches are time-consuming, permitting maximal injection rates in the range of 300-500 cells per hour.

Semi-automated (Eppendorf) and automated (Zeiss) microinjection systems have also been described that include an electronic interface between the micromanipulator and the injection system. Such systems require less technician training and skill, and are thus

preferred to purely manual microinjection approaches. In addition, they enable more rapid injection of cells (> 1,000 cells per hour).

Microinjection has been described for the incorporation of genetic material into adherent cells with some degree of success (85-95% short-term expressing, viable cells, up to 30% stably expressing cells). Manual microinjection of larger adherent cell types (e.g., fibroblasts) has been reported to yield satisfactory survival (85-100%).

Microinjection technology has even been successfully applied to injecting macromolecules into the pronuclei of non-adherent egg cells (100 microns diameter) as part of the protocol used in producing transgenic animals. Such technology has also been used to inject sperm into eggs when performing the intracytoplasmic sperm injection (ICSI) procedure when treating human infertility. However, both the holding pipettes (10 microns) and microinjection needles (1-3 microns) used for these injections are much too large to be useful for injecting smaller cells (eg. hematopoietic cells which are ~5-10 micron diameter).

Small, normally non-adherent cells (e.g., primary hematopoietic cells) manual microinjection has generally yielded only a small percentage of surviving micro-injected cells. For example, in Graessmann, *et al.* (PNAS 77:433-436, 1980), although the Raji human B cell line could be anchored to tissue culture plates coated with anti-lymphocyte IgG, phytohemagglutinin (PHA), or Concanavalin A (Con A), this cell line, due to its small size and fragility, could not be efficiently micro-injected with DNA. Microinjection was only possible when larger polykaryons were formed by cell fusion.

Diacumakos *et al.* (Exp. Cell Res. 1981, 131, 73-77) relate to a method for attaching mouse erythroleukemia (MEL) cells to glass coverslips. This is accomplished by first coating cover slips with collagen, and then subsequently coating the cover-slip with concanavalin A and 1-cyclohexyl-3-(2-morpholinoethyl) carbo-diimide p-toluene-sulfonate methyl ester. Using this approach, the microinjection of inducing chemicals into MEL cells was reported. Since Con A can affect cells in a variety of ways [eg. low doses induce blast transformation and mitosis in normal lymphocytes; higher doses inhibit growth of both lymphocytes and proliferating lymphoid cells (McClain and Edelman, 1976); plating of mitotic HeLa cells onto Con A treated coverslips delays entry into S phase of the cell-cycle (Brown, *et al.*, 1985)], its influence on stem/progenitor cells must be evaluated.

M. Graessmann and A. Graessmann (in *Microinjection and Organelle Transplantation Techniques*. Academic Press Incorporated, London, 1986) describe a general microinjection procedure using glass capillaries (*Methods in Enzymology*; 101:48). These glass capillaries are described as having an outer diameter of about 0.5 microns.

5 Attachment of the Friend leukemia cells, a cell type that typically does not grow in an adherent state, is accomplished through use of concanavalin A and a linker molecule, such as glutaraldehyde. In this manner, attachment of these cells to a plastic Petri dish is reported.

Y. Mori *et al.* (European Patent Application EP0463508 A1) relates to a method

10 for injecting substances into cells using a temperature-responsive polymeric compound and a cell adhesive substance to immobilize the target cell onto a plastic or glass plate. A microcapillary pipette having a diameter of 1 to 2 microns at its tip is employed. The cell adhesive substances included the following molecules: gelatin, lectins, bridge oligopeptides, adhesive proteins, positively charged polymers, collagen, fibronectin,

15 laminin, proteoglycan, glycosaminoglycan and thrombospondin.

There are two primary reasons for the great difficulty in micro-injecting hematopoietic cells, both primary cells [definition: cells directly transferred from an *in vivo* setting (e.g., in human, mouse, etc.) to *in vitro* culture without any transforming event] and transformed cells [definition: cells which have an unlimited potential for further cell

20 division-the transformation event either occurring *in vivo* (e.g., the cells are leukemic) or *in vitro*]: (1) there is significant difficulty in immobilizing hematopoietic cells in a manner that does not significantly affect the biological properties of the attached cells. For example, although attachment of hematopoietic cells has been described via lectins such as PHA, Con A, or pokeweed mitogen, as mentioned above, these agents are well known

25 for their mitogenic, or in some cases inhibitory, effects on hematopoietic cells. Thus, the immobilized cells are likely biologically modified by the immobilization step. (2) the small size of hematopoietic cells, particularly for primary hematopoietic stem cells (5-7 micron diameter), makes them extremely difficult to inject. Since commercially available microinjection needles typically have inner diameters of approximately 0.5 micron- and,

30 therefore, outer diameters in the range of 0.7-1.0 micron- it is not unexpected that hematopoietic cells are adversely affected by injection with such needles. There have been some reports of microinjection needles with outer diameter of 0.1-0.3 micron.

Gene transfer to non-cycling hematopoietic stem cells with acceptable long-term expression in progeny cells continues to be a major technical challenge in the art of gene therapy approaches. Even though a wide variety of methods including electroporation, liposomes, retroviruses, and adeno-associated virus have been described and explored, such  
5 continue to present limitations that preclude the wide-scale, routine use of macro- and micro molecular transfer to living cells. Both electroporation and liposome-mediated delivery have been hampered by the inability to establish stable maintenance of the introduced genes in a significant fraction of cells, while recombinant C-type retroviral vectors and adeno-associated vectors are inefficient in both transduction of non-cycling  
10 cells and maintenance of long-term cell type specific gene expression.

Some have reported retrovirus-mediated genetic modification of cycling human progenitor cells and long-term culture initiating cells (LTC-ICs), these hematopoietic cells reportedly being capable of sustaining hematopoiesis *in vivo* for a finite period (reportedly about 2 to 6 months). For long-term benefit of the gene therapy, this technique likely  
15 requires repeated treatment of the patient with modified stem cell populations, as progeny cells from modified progenitor cells are relatively quickly replaced with unmodified (transduced) progeny of unmodified stem cells.

Clinical trials involving retrovirus-mediated transduction of drug resistance genes (e.g., the human multi-drug resistance-1 gene, MDR-1, which protects cells from several  
20 chemotherapeutic agents such as Vincristine, Taxol and Doxorubicin) into marrow or peripheral blood CD34<sup>+</sup> cells have been reported. The CD34<sup>+</sup> antigen marks all currently assayable human hematopoietic stem and progenitor cells. However, retroviral (type C) vector-mediated transduction requires that the cell be cycling in order to effect transduction. Quiescent, i.e., non-cycling, hematopoietic stem cells, which typically  
25 constitute a portion of the population of cells being treated, are not effectively transduced. Thus, retroviral mediated gene transfer is a fairly inefficient technique for transfer of genetic material into stem cells. Relatively low frequency (0.1-1.0%) of gene marked peripheral leukocytes has been reported using this technique to treat CD34<sup>+</sup> cells for transplantation in human trials. Other studies report that retroviral transduction protocols  
30 used deplete human stem cell reconstituting activity.

The expression of retrovirus-transduced transgenes is frequently silenced in the progeny of transduced human or primate progenitors, and even in progeny of transduced

mouse stem/progenitors (Challita and Kohn, 1994; Akkina *et al.*, 1994; Lu, *et al.* 1994). As a consequence, hematopoietic cells which are retrovirally genetically modified with drug resistance gene may not efficiently display the drug resistance phenotype. This problem, while significant for retrovirus vectors (maximum of 8kb), is even more acute for  
5 adeno-associated virus (AAV) vectors (maximum of 4kb).

A need continues to exist in the medical arts for improved methods of introducing foreign materials into non-adherent cells, particularly into non-adherent cells using microinjection techniques, and the use of these methods in treatment of physiological disorders.

10  $\beta_1$  integrins are members of the integrin gene family of cell adhesion receptors that recognize particular ligands such as fibronectin, laminin, collagen, epiligrin, invasin, and vascular cell adhesion molecule-1 (VCAM-1; Stuijver & O'Toole *Stem Cells* 13: 250-262, October 1995).  $\beta_1$  integrins mediate cell-cell and cell extracellular matrix interactions (Stuijver & O'Toole, 1995). The  $\beta$  subunit of integrins contributes to both ligand binding  
15 and the transduction of intracellular signals. The ligand binding affinity of several integrins from the  $\beta_1(\alpha_4\beta_1, \alpha_5\beta_1, \text{ and } \alpha_6\beta_1)$ ,  $\beta_2(\alpha_L\beta_2 \text{ and } \alpha_M\beta_2)$ , and  $\beta_3$  subfamily  $\alpha_{IIb}\beta_3$  can be regulated by a variety of stimuli (Ref.: Schwartz, M.A., Schaller, M.D., Ginsberg, M.H. (1995) *Annu. Rev. Cell Dev. Biol.* 11:549-599. (Integrins: Emerging Paradigms of Signal Transduction)). In turn, the integrins can also affect different cellular functions. For  
20 example, monoclonal antibodies directed against the  $\beta_1$  subunit induce negative signaling effects on T cell proliferation (Schwartz *et al.*, 1995).

Various cell types express cell-surface molecules of the integrin family which are used in attachment of cells to various substrates including extracellular matrix molecules and other cells. Several methods have been reported for activation of cells expressing  
25 integrins to attach to particular substrates. These methods include incubation of cells with anti-integrin antibodies, cytokines, extracellular matrix proteins (e.g., fibronectin) and peptides or fragments thereof, lipids, divalent cations, and phorbol esters.

A variety of primary and transformed cell lines have reportedly been immobilized to surfaces via integrin activating antibodies. The primary cells have included PHA-stimulated peripheral blood T-cell blasts (Wayner and Kovach, 1992) and resting peripheral  
30 blood lymphocytes (Arroyo, *et al.*). Transformed cell lines that have reportedly been immobilized include K562 (human erythroleukemic; Kovach *et al.*, 1992), U937 (human

myelomonocytic; Wayner & Kovach), M07e (Levesque), TF1 (human erythroleukemic; Levesque), A375 (melanoma cells; Arroyo *et al.*), Jurkat (human T lymphoblastoid; Wayner and Kovach, 1992), Ramos (human B lymphoblastoid; Wayner and Kovach, 1992), and ST-1 (human B lymphoblastoid; Wayner and Kovach, 1992).

- 5           While some investigators have reported the ability of anti-integrin antibodies and other agents such as peptides to activate a cell to become adherent and to thus adhere to a substrate, there has been no application of this technology to microinjection of cells that do not grow in an adherent state, such as hematopoietic cells. This technical difficulty, among others, has precluded the use of microinjection of important cell types that do not
- 10       grow in an adherent state, most notably hematopoietic stem cells. The use of cell-surface integrins to immobilize hematopoietic cells would overcome this difficulty and allow these cells to be micro-injected.



DISCLOSURE OF THE INVENTION

In one aspect, the present invention provides for improved methods of immobilizing non-adherent cells to a surface sufficient to facilitate the efficient introduction of macro- and micro-molecules, including genetic material, proteins, peptides and immunoglobulins into living cells. The present invention contemplates that a variety of immobilization techniques can be employed for immobilizing a non-adherent cell to a surface. In the present invention, a non-adherent cell is defined as: 1) a cell that is routinely maintained in suspension culture in vitro; or 2) a cell which is routinely maintained in an adherent state in culture, but is intentionally detached and allowed to be maintained for a defined period of time in suspension for the purpose of experimentation or manipulation.

In some embodiments, the method comprises attaching non-adherent cells having cell surface-expressed adhesion molecules, such as integrins (Fig. 1A & 1B), to a surface, the method comprising: treating said surface with adhesive molecules, and treating said non-adherent cell having cell surface-expressed molecules such as integrins or CD44, with an activating molecule such as an antibody which activates the cell surface expressed molecules to bind to a surface treated with adhesive molecules. This procedure results in an otherwise non-adherent cell being attached to a substrate surface sufficient for performing a microinjection procedure (Fig. 1B).

In some embodiments the non-adherent cells can also be attached to a surface comprised of adhesive molecules without the use of activating antibodies (Fig. 1C). This depends on the cell type and the adhesive chosen. The present invention, in particular aspects, provides for immobilization of cells to substrates [e.g., fibronectin or portions of the fibronectin molecule derived either by protease digestion, recombinant expression (eg. Retronectin; CH-296), or synthesized peptides] without additional stimuli of cell surface expressed adhesion molecules, i.e., attachment of some cell types to fibronectin peptides/fragments may be sufficient for microinjection without prior activation (e.g., U937, HUT-78 cells, CEM cells, hematopoietic stem/progenitor cells). Some cell types (e.g., hematopoietic stem/progenitor cells) attach to the carboxyl end of fibronectin with sufficient strength to withstand microinjection.

Examples of adhesive molecules include by way of example and not exclusion: fibronectin, collagen, laminin, epiligrin, invasin, osteospondin, thrombospondin,

proteoglycan, glycosaminoglycan, ICAM and VCAM-1, and fragments thereof derived by protease digestion or recombinant expression; synthesized peptides (with or without chemical modification), or oligosaccharide fragments thereof. Such is also anticipated to include both linear and circularized fragments of said proteins and peptides, or fragments thereof.

In another aspect, the invention provides for a method for the delivery of material into a non-adherent cell. In one embodiment, the method comprises: immobilizing the non-adherent cell to an adhesive surface, and micro-injecting a solution containing said foreign material into said immobilized non-adherent cell through a microinjection needle (Fig.12B). The microinjection needle may be further described as having an outer diameter in the range of about 0.05 to about 1 micron, or about 0.05 to about 0.5 microns.

In other embodiments, the method of immobilizing non-adherent cells to a surface comprises: immobilizing the non-adherent cell with a holding pipette having a distal end with an inner bore diameter of about 0.5 to about 2.5 microns; and applying a vacuum with a syringe assembly to the holding pipette, driven either manually (e.g., Eppendorf or Zandler) or with a powered device (e.g., World Precision Instruments), to said holding pipette while said distal end of said holding pipettes is in close proximity to said non-adherent cell (Fig.12A). In some embodiments, the holding pipettes may be made using a DeFonbrune microforge. According to one method for preparing the pipettes, appropriate bends are made in a glass capillary so that it fits in a chuck assembly holder, such that a 1-5 gram weight can be hung from the capillary positioned inside a heating filament. Heat is then applied softening the glass and resulting in the weight pulling the glass capillary to an about 1 to an about 5 micron diameter, at which point the piece of glass capillary from which the weight is suspended breaks away. This results in a holding pipette with an about 1 to an about 5 micron diameter tip. The tip is then brought close to the heating filament, and the tip is heat polished. This provides a smooth tip with an opening of an about 0.5 to an about 2.5 microns. This holding pipette can then be attached to a syringe assembly that can be used to create a vacuum that will hold the cell in place for the microinjection procedure.

When making such a holding pipette, one is not limited to the DeFonbrune microforge. For example, any of the commercially available needle pullers (Sutter Instruments, Narishige, Kopf) can be used to pull a capillary to provide a pipette as

described here as part of the invention. For most embodiments of the invention, the tip should be heat polished with a microforge (DeFonbrune or Narishige).

5 The microinjection method of the invention is directed at providing a population of modified non-adherent cells, especially hematopoietic stem cells that include a desired molecule without significant loss of living cells or biological function. The estimated number of stem cells that will need to survive the microinjection procedure for a gene therapy procedure to is estimated to be about 150 to about 5,000 stem cells.

10 The present invention also contemplates that microinjection-mediated delivery of transgenes or transgenes with accessory proteins, such as integration proteins, directly to the nuclei of primitive cord blood stem cells (Fig. 8), will be more efficient than delivery of same with retroviruses and AAV vectors. It is further contemplated that larger regions of DNA, such as those containing regulatory elements and intron/exon structure, will be deliverable by the present microinjection method. Such will also avert the dysregulated expression and silencing frequently observed in the progeny of transduced stem cells.

15 The present invention also contemplates using other methods for delivery of transgenes or transgenes with accessory proteins. For example, iontophoresis, particle bombardment, electroporation, cationic liposome-mediated transfection, peptide-mediated gene delivery (e.g. polylysine), receptor-mediated endocytosis, red blood cell-mediated transfection, hypotonic swelling, micropicking, and addition of nucleic directly to the medium surrounding the cells.

20 In another aspect, the invention provides a gene therapy method for treating physiological disorders (Figs. 5, 6, & 7). By way of example, such physiological disorders include thalassemia and cancer.

25 Another aspect of the invention provides a specifically designed apparatus for delivering foreign material into a non-adherent cell. In some embodiments, the apparatus comprises a microinjection needle having an outer diameter in the range of about 0.05 to about 0.5 microns.

30 In some embodiments, the apparatus comprises a microinjection needle having an outer diameter in the range of about 0.05 to about 0.5 microns with a beveled tip. Internal opening dimensions may be employed that are tailored to accommodate the thickness of the walls of the glass capillary used in making the needle in some embodiments of the present invention. In particular, the needle will be made using a glass capillary with a sufficient

5 wall thickness that when used in making the injection needle will result in a needle that has a tip with thick enough walls to not easily break when coming in contact with a plastic material when the needle is used in a cell injection procedure. In some embodiments, the needle will satisfy the above requirements and when used in the injection procedure results in minimal damage to the cells, i.e., will have the best observed cell viability following the injection procedure.

10 In some embodiments of the injection needles provided in the present invention, the glass capillary will have a sufficient wall thickness such that the needle tip will have a thickness such that it does not easily break when coming in contact with a material more pliable than plastic. The needle tip may be further defined as being coated with a non-sticky compound (e.g., silicon).

15 These embodiments will provide a needle that will not stick to intracellular components, thus decreasing the chance of damaging the cell via the microinjection procedure. The needle in other embodiments may be coated both externally and internally with a non-sticky compound (e.g., silicon). These needles will be particularly efficacious in the delivery of viscous, concentrated, or sticky macromolecules from the needles, in particularly those needles with very small outer diameters (.05- 0.5 microns).

20 The invention, in still another aspect, provides a method for the protection of hematopoietic stem cells and their progeny from chemotherapy. Such cells, by way of example, will be treated such that they gain resistance to such chemotherapy agents as alkylating agents, anthracyclines, vinca alkaloids, Etoposide, Taxol and the like, or any combination. The protection of the hematopoietic stem cell and its progeny from chemotherapy will be provided by microinjection of said cells with multiple drug resistance genes, such as the MGMT gene and/or MDR-1 gene, and delivery of these modified cells to a patient. The MGMT gene has been isolated and described in the literature, which teachings are specifically incorporated herein by reference (Wang, *et al.*, 1996; Moritz, *et al.*, 1995; Preuss, *et al.*, 1996). The MDR-1 gene is also described in the literature, and in particular in I. Roninson, *et al.*, which reference is specifically incorporated herein by reference. (Koc, *et al.*, 1996).

30 Preparations comprising a population of cells enriched with modified human hematopoietic stem cells having nucleic acid material introduced into them comprise yet another aspect of the invention.

Kits useful for the immobilization and microinjection of foreign materials into non-adherent cells are also provided. These kits in some embodiments comprise: a carrier means adapted to contain at least two container means; a container means comprising a microinjection needle having an outer diameter in the range of about 0.05 to about 1  
5 microns, and a container means comprising a microinjection needle having all of these characteristics. The kit can comprise an immobilization surface (available both uncoated or adhesive-coated), microinjection needle(s) and, optionally, a cloning ring. The adhesive when not coated directly on the immobilization surface can be provided in a separate container. When required, activators of cell attachment (e.g., antibodies that activate cell  
10 surface integrins) can be provided in a separate container. The said kit may also include reagents (eg. divalent cations, peptides, etc.) used for the detachment of cells from the adhesive after microinjection.

It should be understood that application of the methods and apparatus provided by this invention as opposed to those disclosed by the prior art will (1) facilitate  
15 microinjection of a wider range of primary and transformed non-adherent cells; and (2) permit immobilization; microinjection and recovery of micro-injected cells with less adverse effects upon the biological activity (e.g., *in vivo* reconstitution of the hematopoietic system by genetically modified stem cells) of the cell; and (3) permit injection of non-adherent cells with semi-automated or automated microinjection instruments capable of  
20 injecting more cells per hour.

In another embodiment, the present invention provides a kit for the microinjection of foreign material into non-adherent cells having cell surface-expressed integrins, the kit comprising: an immobilization surface coated with an adhesive, and anti-integrin  
25 monoclonal antibody (or other equivalent integrin activation) and a microinjection needle having an outer diameter in the range of about 0.1 to about 0.15 microns. Other embodiments of the kit may include microinjection needles with other outer and inner diameter sizes, or combinations of various sizes, as described herein.

It is contemplated that the methods and apparatus disclosed herein will be useful for the transfer of foreign material into human cells as an approach to gene therapy and  
30 somatic cell therapy.

Thus, in accordance with one aspect of the present invention, there is provided a composition comprising hematopoietic stem cells, or more generally non-adherent cells,

which are genetically engineered with DNA which encodes a therapeutic agent of interest. The genetically engineered cells are subsequently employed as a therapeutic agent.

Accordingly, the present invention also provides a gene therapy method for the treatment of physiological disorders responsive to gene therapy comprising: administering  
5 to a patient parenterally a composition enriched for genetically modified human hematopoietic stem cells, the stem cells containing genetic material delivered by microinjection (Fig. 7). It is contemplated that a composition of cells that includes at least about 150 to about 5,000 viable genetically modified cells, potentially supplemented with a sufficient number of genetically modified or unmodified hematopoietic progenitor cells  
10 for short-term hematopoietic reconstitution, will provide treatment and or therapy of the targeted disorder.

In accordance with another aspect of the present invention, there are provided hematopoietic stem cells, which have been genetically engineered by microinjection to include DNA which encodes a marker or therapeutic agent, with such cells expressing the  
15 encoded product *in vivo*. In this particular embodiment, the DNA cures a genetic deficiency of the cells and the expression product is either not secreted from the cells (for example, hemoglobin) or secreted from the cells (for example, Factor VIII).

The invention is also directed to a method of enhancing the therapeutic effects of hematopoietic stem cells (hSCs).

20 In accordance with another embodiment, there is provided a process for treating a patient with a therapeutic agent by providing the patient with hSCs genetically engineered with DNA encoding such therapeutic agent.

In accordance with another embodiment, a patient is provided with hSCs which are genetically engineered with DNA encoding a therapeutic agent whereby such therapeutic  
25 agent may be expressed *in vivo*. As hereinabove indicated, such genetically engineered cells may be provided by administering to the patient hSCs which have been genetically engineered *ex vivo* by hSCs microinjection.

In accordance with a further aspect of the present invention, there is provided a composition comprising (i) hSCs genetically engineered with DNA encoding a therapeutic  
30 agent and (ii) a pharmaceutically acceptable carrier suitable for administration to a patient.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A and FIG. 1B. FIG. 1A demonstrates potential mechanisms for activating the integrins present on the surface of various non-adherent cells. In FIG. 1A, CD34<sup>+</sup> stem/progenitor cells are shown to express both the  $\alpha_4 \beta_1$  integrin as well as the  $\alpha_5 \beta_1$  integrin on their surface. The adhesive molecules used as an example here is fibronectin. The  $\alpha_4 \beta_1$  is shown to bind to the LDV-containing peptide sequences, while the  $\alpha_5 \beta_1$  binds to the RGD-containing peptide. The cells shown with a "rounded" suspension morphology when they attach to a substrate, such as fibronectin, are generally attached only in a weak tethered form. The tethering of cells to a substrate is not sufficient for microinjection of cells employing a rapid scale method because they are easily dislodged by the microinjection needle. The activation of integrins by various mechanisms including those enumerated here leads to very tight attachment of cells to fibronectin by converting integrins from a state with low affinity for the ligand to a state with high affinity (Fig. 1A) for the ligand, and to support the spreading of the cells. Cells that are more avidly bound do withstand the semi-automated and automated microinjection process. Fig. 1B demonstrates that, in some cases, attachment and spreading of a cell on an adhesive surface can occur without additional activating agents added prior to, or concurrent with cell attachment. FIG. 1C demonstrates the ability of cells to attach and spread, in the absence of any activating agent to carboxyl terminal fragments of fibronectin (containing the RDV and LDV recognition sites). (Kimizuka et al., 1991).

FIG. 2A and 2B. Methods by which cells immobilized on a surface may be released from the surface immobilization. Cells immobilized on a surface with fibronectin (Fig. 2A) may be released with a variety of methods including competition with peptides, inhibition of integrin mediated attachment by calcium, chelation, trypsinization, or disruption by pipetting. The cells can eventually be recovered in a non-adherent form where they lose their spread, adherent morphology and return to a more rounded, non-adherent shape (Fig. 2B).

FIG. 3 shows retention of both myeloid and erythroid colony forming activity by CD34<sup>+</sup> cells that have been immobilized via an activated integrin/fibronectin attachment (forward slashed bar). Colony forming activity by concanavalin A immobilized CD34<sup>+</sup> cells is also shown (back slashed bar). Shown on the left is erythroid burst (BFU-E) colony

formation. Both methods of attachment showed no significant difference from that of the control non-immobilized cells (open bar). Myeloid (CFU-GM) colony formation showed no significant decline in colony formation for the integrin/fibronectin immobilized cells in comparison with the control. In contrast, with concanavalin A immobilization, the myeloid colony formation was only about 50% of the control non-immobilized cells. The small standard deviation indicates this is a significant difference both from that of the control and the integrin/fibronectin sample.

FIG. 4 shows a time course evaluation of the number of fluorescent cells as a percentage of micro-injected cells. CD34<sup>+</sup> cells (—■— and —▼—) or CD34<sup>+</sup>/CD38<sup>+</sup>/Thy-1<sup>lo</sup> cells (—▲— and —●—) were attached with the integrin/fibronectin method outlined in the examples. Cells were injected with needles having 0.2 micron O.D. tips and FITC-Dextran material of 150,000 molecular weight was injected. Shown at the less than 1 hour time point is the assessment of the percentage of good injections giving rise to fluorescent cells immediately after injection. These samples were then followed for various time points, 4 hours, 24 hours, 48 hours or greater after the injection and the number of cells that were fluorescent is shown as the percentage of the total number of good injections. The decline in this number reflects: (1) quenching of fluorescence by subsequent fluorescent microscopy; (2) death of cells due to microinjection-mediated injury; and/or (3) possible toxicity of FITC-Dextran for hematopoietic stem/progenitor cells. Shown are 2 representative studies for each cell type.

FIG. 5 shows a schematic of the hematopoietic system with all of the various lineages proceeding eventually from the pluripotent stem cells. The CD34<sup>+</sup> cells containing all measurable human stem/progenitors constitute 0.5 to 1% of the total mononuclear cells in cord blood or bone marrow. Also shown is a phenotype that was used to describe the pluripotent stem cell. Those are cells that are CD34<sup>+</sup>, CD38<sup>-</sup>, CD45Ra<sup>-/lo</sup>, CD71<sup>-/lo</sup>, Thy-1<sup>lo</sup> and a rough estimate of their frequency is expressed as a percentage of mononuclear cells.

FIG. 6 shows, in schematic form, the complete hematopoietic system of individuals with defective genes, deletions or mutations in their chromosomal DNA (\* = mutation in chromosomal DNA sequences).

FIG. 7 shows the outcome of the introduction of genetically modified stem cells into the patient (\* = mutation in chromosomal DNA sequences; ■ = corrected gene). If



the correcting DNA is put into the pluripotent stem cell and the introduced DNA is retained in progeny cells as the hematopoietic system is allowed to develop, genetically corrected cells will comprise the complete hematopoietic system (i.e., all lineages and stages of maturation).

5           FIG. 8 shows an advantage of the microinjection system—that is, the ability to co-deliver DNA (rectangles) together with proteins (circles) into the same cell. In this example, the proteins were chosen to facilitate the integration of the DNA sequences into the chromosomal DNA. Shown here are examples where the sequences and proteins are selected either from a murine leukemia virus (a retrovirus) or the adeno-associated virus.

10           FIG. 9 shows the DNA sequences which are intended to correct a genetic defect (\*) in the chromosomal DNA, and in this case is proposed that proteins (circles) active in homologous recombination will be co-injected together with the correct DNA sequences. The object is to replace the deleted or mutated sequences with their correct copies supplied via microinjection—eventually giving rise to a cell which would be corrected at the  
15           previously defective allele.

          FIG. 10A and FIG. 10B show CD34<sup>+</sup> stem progenitor cells plated onto fibronectin in the absence of any antibodies (i.e., simply in media, FIG. 10A) and via their attachment to fibronectin in the presence of the anti  $\beta_1$  activating integrin antibody, TS 2/16.2.1 (FIG. 10B). As can be seen from FIG. 10A, cells attached in the absence of antibody are only  
20           loosely tethered. They maintain their round morphology, and the refractile nature of the outside surface of the cells indicates that they are not attached flat to the surface; rather they are simply tethered at a point. This is to be contrasted from the extremely flat morphology of the integrin attached cells (FIG. 10B). These cells are tightly attached to the surface, frequently have podia emanating from the cell, and are highly spread in  
25           comparison with either the non-immobilized or tethered cells.

          FIG. 11 shows an ethidium bromide stained agarose electrophoresis gel of lambda wild type DNA digested with HindIII/EcoRI either pre- or post-filtration through a 0.1 micron filter. There was no evidence for loss of material of sizes at least up to 21 kb in length. Since all fragments (including the 21kb fragment) successfully passed through  
30           filter pores of 0.1 micron size; this strongly suggests that linear fragments of at least 21 kb in length will successfully pass through injection needles of inner diameter greater than or equal to about 0.1 micron inner diameter.

FIG. 12A and FIG. 12B - Microinjection Approaches. FIG. 12A demonstrates an injection technique employing a holding pipettes to stabilize the cell. Once stabilized with the holding pipette, (via vacuum suction) the cell may be injected with the microinjection needle. The microinjection needle is represented by the needle having a label of 0.12 $\mu$ . The holding pipette is represented by the appendage marked with a 2-4 $\mu$ . (Both of these dimensions (i.e., 0.12 $\mu$ , 2-4 $\mu$ ) are chosen for example purposes only). FIG. 12B represents an injection technique for microinjection of cells that are immobilized by treating a surface of a culture plate with an adhesive molecule. Cells attached through the methods described in this application withstand the microinjection process.

FIG. 13 - Attachment/detachment/spreading of U937 cells on commercially available fibronectin-coated dishes following treatment with 1mg mAb TS2/16.2.1/ml (-■- = cells attached; -◆- = cells with micropseudopodia, projections or extensions; -●- = cells loosely attached).

FIG. 14 - Proliferation of U937 cells following attachment to commercially available fibronectin coated dishes (-■- = U937 cells treated with 1mg mAb TS2/16.2.1/ml; -◆- = U937 cells untreated) viability U937 cells treated with mAb TS2/16.2.1 = 96.4%; U937 cells untreated = 95.8%.

FIG. 15 - Attachment and spreading of CD34<sup>+</sup> cells isolated from cord blood on commercial coated fibronectin dishes following treatment with TS2/16.2.1 mAb (-■- = cells attached; -◆- = cells with micropseudopodia, projections or extensions; -●- = cells loosely attached).

FIG. 16 - Proliferation and viability of CD34<sup>+</sup> cells isolated from cord blood in the presence or absence of 1mg mAb TS2/16.2.1/ml. (-■- = presence of mAb; -◆- = presence of control IgG mAb). Viability of CD34<sup>+</sup> cells treated with mAb TS2/16.2.1 = -●-; untreated = -▲-.

FIG. 17 - CD34<sup>+</sup> cell attachment to fibronectin (-■- and -◇-) versus retronectin (-○- and -▲-) in the absence (-◇- and -▲-) or presence (-■- and -○-) of 1mg mAb TS2/16.2.1/ml.

FIG. 18 - U937 cells quartz/borosilicate needle. Percent survival U937 cells 2 hours, 24 hours, and 48 hours after injection with the quartz/borosilicate needle (hatched bars = quartz; open bars = borosilicate).

FIG. 19 - CD34<sup>+</sup> cells quartz/borosilicate needle. Percent survival of cells at 2 hours, 24 hours, and 48 hours after injection (hatched bars = quartz; open bars = borosilicate).

FIG. 20 - Size distribution data for borosilicate needle version 1.0B; frequency  
5 versus outer diameter (in microns) of borosilicate needle.

FIG. 21 - Size distribution data for quartz needle version 1.0Q. Frequency versus  
outer diameter (in microns) of quartz needle.

FIG. 22A, FIG. 22B, & 22B' - FIG. 22A - flow chart demonstrating loading of a  
siliconized needle. Use of a pressure device to load a sample into the needle barrel of a  
10 siliconized needle. FIG. 22B & B' - air or other gas bubble is expelled from the tip of the  
silicon needle after loading of the sample. This may be accomplished by use of a glass, or  
other similar material, filament to the tip of the sample-loaded needle.

FIG. 23 - gridded cell plate. An elastomeric stamp or manifold device may be used  
to lay down defined islands of adhesive material in the desired spacial orientation to  
15 accommodate the number of cells to be injected in a single batch. By way of example, the  
defined islands of adhesive may be laid down with a pre-determined spacial orientation to  
accommodate about 1,000 cells. Each cell will be spaced every about 10 to about 20  
microns. In that example, the stamp will have an overall dimension of 1mm x 1mm. In  
some embodiments, the stamps may be arranged to conform to the organization of the  
20 needles to be used in the injection procedure. One or more needles may be configured to  
move from cell to cell, with one needle injecting the cells in each of the 1mm islands. In  
some embodiments, the geometry of the surface area covered by the adhesive material can  
be controlled to limit cell spreading in order to regulate cell function (eg. proliferation,  
differentiation, secretion of cell products, etc.).

FIG. 24A-24B - Injection manifold. FIG. 24A demonstrates the top view of one  
25 embodiment of the injection manifold. The injection needles 1 extend from the manifold  
2. The number of needles and spatial arrangement of the needles may vary according to  
the desired arrangement of the user and to provide a needle injection profile that would  
correspond to the grid pattern arrangement of the gridded cell plate wells used. FIG. 24B  
30 demonstrates a side view of the injection manifold. An inlet 3 is connected to the manifold  
2. A single or multiple needle arrangement of needles 1 extend from the manifold 2.

FIG. 25 - Microinjection assembly unit. The needle 1 is positioned to provide contact with a tissue culture dish 5. Metal filaments 2 contact a resistivity monitor 4, which is in turn in contact with a sound system 3.

FIG. 26 - Viability of U937 cells after microinjection using borosilicate (hatched bars) or quartz needles (speckled bars), versions 1.0B and 1.0Q, respectively.

FIG. 27 - Viability of CD34+ cells after microinjection using borosilicate (hatched bars) or quartz needles (speckled bars), versions 1.0B and 1.0Q, respectively.

FIG. 28A & FIG. 28B - Scanning electron micrographs of A. Version 1.0B borosilicate injection needle. FIG 28 B. Version 1.0 Q quartz injection needle.

### BEST MODES OF CARRYING OUT THE INVENTION

The microinjection method practiced according to the invention in some embodiments employs non-adherent cells and microinjection needles with outer diameters of about 0.05 microns to about 0.5 microns. The method in some applications provides for immobilization of a non-adherent cell onto a surface, followed by microinjection of the cell to include a desired foreign material. The invention further provides for the removal of modified cells from a culture surface with minimal damage and/or loss of cell viability.

The modified cells as provided according to the present invention can be used in a variety of applications, including: (a) in laboratory studies, (b) for production of desired proteins (e.g., *in vitro* production of monoclonal antibodies), and (c) to treat a physiological disorder. In this regard, the techniques disclosed herein may be used in gene therapy. In another aspect, the invention provides for a preparation of cells enriched in genetically modified cells. Such preparations may also be used to administer parenterally to a patient suffering from a gene therapy responsive physiological disorder wherein the genetically modified non-adherent cell and its progeny may express a therapeutic agent, thus treating the patients physiological disorder.

#### **Microinjection Method**

As used herein, the terms "microinjection" or "micro-injecting" refer to the delivery of foreign material into a non-adherent cell. In some embodiments, this method employs a microinjection needle as described below.

In some embodiments of the method, the microinjection process of the invention proceeds generally as follows. A cloning ring is affixed to an immobilization surface, such as a tissue culture plate, and the surface of the plate enclosed by the cloning ring is coated with an adhesive, such as fibronectin. The coated immobilization surface is then exposed to a mixture of non-adherent cells to be immobilized in the presence of an activating anti-integrin monoclonal antibody for a period of time and at a temperature sufficient to permit immobilization of the non-adherent cells to the immobilization surface. In other instances, the non-adherent cells will be added to the said immobilization surface in the absence of an activating antibody or other activator of attachment. An ideal needle is loaded with a sample solution containing an effective amount or concentration of foreign material. The tissue culture plate containing the immobilized non-adherent cells is placed on the stage of a microscope, and the microinjection needle is placed in a micromanipulator such as that sold by Narishige, or an electronically controlled manipulator such as that sold by Eppendorf, mounted on the same microscope. A device, such as an SAS 10/2 air screw actuated microinjection/aspiration syringe or an automated Eppendorf 5246 Transjector, provides the pressure necessary for delivery of the sample solution (possibly containing a fluorescent marker) from the microinjection needle into the nucleus of the cell. Following insertion of the microinjection needle into the nucleus of the non-adherent cell, an effective amount of the sample solution is injected into the cell. Delivery may be monitored *via* a phase contrast microscope. However, it may be advantageous to deliver such small volumes that no observable change in the cell will be detected *via* light microscopy. After retraction of the microinjection needle, nuclear delivery of the foreign fluorescent material may be confirmed with a microscope equipped with a fluorescence detector and the modified cells are subsequently detached from the immobilization surface.

The microinjection process can be done manually, semi-automatically, or automatically according to the equipment employed. The manual microinjection approach involves using a micromanipulator to direct a glass micropipette (loaded with an injection sample) into a living cell's nuclear or cytoplasmic compartment, all viewed with a phase-contrast microscope. The injection needle is connected to a syringe assembly that provides the pressure which continuously forces the sample out of the needle. The needle tip is inserted into the cell, and the lightening of the phase contrast caused by the flow of sample solution into the cell is visually monitored. The change in phase contrast indicates

injection of the sample into the cell, whereupon the injection needle is removed from the cell. The semi-automatic microinjection process proceeds as follows: The microinjection needle is directed into the nucleus of the cell using the manual settings. This is done to set the Z value, i.e., the vertical position that the needle will return to when performing the automatic injections. Upon setting the Z value, the needle is pulled out of the nucleus and positioned above the nucleus of the cell to be injected. The automated system is then activated. The needle is directed into the cell nucleus at the same time a pulse of pressure expels the injection sample into the nucleus. After the injection is completed, the needle returns to the position directly above the injected cell. A new cell is then located and the procedure repeated.

The volume of solution containing foreign material which is micro-injected into the non-adherent cells can be optimized as desired. Generally, the volume injected will not exceed about 2% to about 5% by volume of the non-adherent cell nucleus receiving the solution. The concentration of foreign material and the physical properties of the solution being injected into the non-adherent cell can impact the success of the microinjection. According to the invention, other variables such as temperature, speed of microinjection needle penetration into and retraction from the non-adherent cell, inner and outer microinjection needle tip diameter, length of time the needle has an increased internal pressure (for expelling the injection sample), angle of needle when penetrating the cell, and the internal pressure in the needle both during and after the injection procedure must be optimized for each cell type other parameters possibly requiring optimization. The temperature used during microinjection can be varied from about room temperature (22° C) to about 37°C using the heated stage that is part of the microscope used for the injection procedure. The volume of solution injected into the non-adherent cell will vary, among other things, according to the volume of the non-adherent cell.

The pressure used to micro-inject sample solution into the cell will vary according to inner diameter and tip geometry (e.g., taper length and flare) of the microinjection needle and sample solution concentration and viscosity. The pressure should be sufficiently low to maintain sample solution flow rate and delivered volume below that which is determined to provide maximum cell viability following the injection procedure.

As used herein, the term "microinjection needle" is taken to mean a microcapillary comprised of borosilicate, alumina silicate, or quartz glass, or other suitable material,

which is used to inject foreign material into non-adherent cells. The microinjection needles of the invention can be prepared from conventional glass capillaries with an automated pipette puller such as the P-87, P-97, or P2000 models from Sutter Instruments. The microinjection needles can also be prepared manually by employing a microforge or other  
5 similar device. The microinjection needles of the invention will have an outer diameter less than about 0.5 microns, preferably less than about 0.25 microns and more preferably in the range of about 0.05 microns to about 0.5 microns. The inner bore of the microinjection needles will be sufficiently large enough to permit passage of macromolecules from a reservoir through the microinjection needle tip and into the non-  
10 adherent cell and will generally be about 0.02-0.4 microns in diameter.

The microinjection needles contemplated by the present invention can be prepared generally by optimizing the particular heating filament, the type of capillary (glass composition and inner/outer diameter), and equipment settings (e.g., heat, pull strength, and velocity of pull) employed. The final size and geometry of the microinjection needle  
15 can be determined by a variety of methods, such as scanning electron microscopy (SEM), resistivity measurement, or bubble pressure assay.

The final tip outer diameter (O.D.), taper length, and flare of the microinjection needle can be controlled by employing the method described herein. Considerations such as filament or laser temperature, size of filament, velocity of pull, pull rate, initial capillary  
20 outer diameter, initial capillary wall thickness, initial capillary bore inner diameter (I.D.), capillary composition, period of exposure to heat and annealing rate can all be optimized as needed to yield microinjection needles having the desired characteristics.

When practiced according to the present invention, a non-adherent cell, having been micro-injected with a solution containing a foreign material, will remain viable for an  
25 extended period of time and will retain and possibly express the foreign material, i.e., a non-adherent cell receiving foreign DNA will be able to replicate the DNA and express a protein associated with that DNA.

The process of the present invention is not limited to microinjection of foreign materials solely into the nucleus of non-adherent cells. For example, the foreign material  
30 can be injected into the cytoplasm or various other cellular organelles.

As used herein, the term "foreign material" refers to materials such as intact virions, DNA, RNA, proteins, small organic molecules, metabolites, macromolecules, organelles,

plasmid vectors, enzymes, inorganic substances, chromosomes, artificial chromosomes, episomal plasmids and other materials which are external to the non-adherent cell being micro-injected.

5 A "non-adherent cell" is one that grows as a suspension culture as opposed to one that grows attached in tissue culture. Examples of non-adherent cell types contemplated by the present invention include mouse, human, primate and canine cells such as primary and transformed B-cells (B-lymphocytes), T-cells (T-lymphocytes), hematopoietic stem cells, granulocytes/neutrophils, myeloblasts, erythroblasts and others. Primary stem/progenitor cells contemplated in the invention also include CD34<sup>+</sup>, CD34<sup>+</sup>/CD38<sup>-</sup>,  
10 CD34<sup>+</sup>/CD38<sup>+</sup>, CD34<sup>+</sup>/lin<sup>-</sup>/Thy-1<sup>lo</sup>, CD34<sup>+</sup>/CD45Ra<sup>-lo</sup>/CD71<sup>-lo</sup>/Thy-1<sup>lo</sup>, CD34<sup>+</sup>/c-kit<sup>lo</sup>, CD34<sup>+</sup>/CD38<sup>-</sup>/CD33<sup>-</sup>/CD19<sup>-</sup>/CD45Ra<sup>+</sup>/c-kit<sup>+</sup>, CD34<sup>+</sup>/CD38<sup>-</sup>/CD45Ra<sup>-lo</sup>/CD71<sup>-lo</sup>/Thy-1<sup>lo</sup>, and CD34<sup>+</sup>/CD38<sup>-</sup>/Thy1<sup>lo</sup>. Transformed hematopoietic cells useful in the invention include a variety of cells such as U937 and KG-1. A primary cell is one which is directly removed from its *in vivo* source; i.e., the cell has not been manipulated or transformed to provide for  
15 indefinite growth in culture.

In practicing the microinjection method of the invention, the following four criteria should be considered: (a) a non-adherent cell should be attached sufficiently so as to minimize dislodgement due to microinjection, (b) the micro-injected non-adherent cell should be removable from the surface to which it is attached with minimal damage or  
20 reduction in biological activity, (c) the immobilization should generally not induce cell activation and/or differentiation. Such could potentially interfere with subsequent biological activity of the non-adherent cell, e.g., loss of stem cell activity. An additional consideration is that the microinjection process itself should not adversely impair the viability or biological function of the cell.

25 As used herein, the term "immobilization surface" is taken to mean a plastic, glass, quartz or other surface onto which a non-adherent cell can be immobilized or attached. Such surfaces may include slides, Petri dishes, plastic/tissue culture plates or dishes, coverslips, chromatographic resins, porous membranes, holding pipettes and the like.

By "immobilizing" or "immobilization" is meant the process by which a non-  
30 adherent cell is attached to or held by an immobilization surface with sufficient strength to permit microinjection. Such processes include the retention of a non-adherent cell by a holding pipette having a reduced pressure or vacuum, attachment of the non-adherent cell



to a surface by way of lectins or a linking agent, attachment of the cells to a surface via antigen-specific monoclonal antibodies either directly or indirectly, or activation of cell surface expressed adhesion molecules such as integrins or other adhesion proteins on the non-adherent cell by way of various activating agents, activating anti-integrin antibodies, 5 cytokines, or activating cations to include divalent cations (e.g.,  $Mn^{++}$ ). Alternatively, cells expressing adhesive molecules such as integrins may not require additional activation for strong attachment. A non-adherent cell attached to or held by an immobilization surface is referred to as "an immobilized non-adherent cell."

By "lectin" is meant materials such as PHA and Con A. Lectins are plant and 10 animal proteins known to interact with specific carbohydrate structures on the surface of cells, thereby, facilitating attachment. By "linking agent" is meant materials such as glutaraldehyde and collagen.

As used herein, the terms "substrate" or "adhesive" are taken to mean materials such as fibronectin, collagen, laminin, VCAMs, ICAMs, epiligrin, invasin, osteospondin, 15 thrombospondin, hyaluronic acid, proteoglycan, glycosaminoglycan, or fragments thereof (to include recombinant molecules) or peptides (unmodified or chemically modified). Cell surface expressed integrins on non-adherent cells bind with the substrate or adhesive either in the native state or once the integrin has been activated with agents such as anti-integrin monoclonal antibodies. The substrate or adhesive attaches directly to an immobilization 20 surface (or in some cases a linker is used to attach the matrix molecule to a particular surface) thereby permitting immobilization of a non-adherent cell onto the immobilization surface. When the adhesive is attached to the immobilization surface, the resultant is termed an "adhesive-surface couple".

Integrins are proteinaceous molecules expressed on the surface of various cells and 25 serve a variety of biological functions such as mediating adherence to various matrices/cells. A cell surface expressed integrin is an integrin that is produced by a cell and is associated with the cell membrane or cell surface and generally has at least a part of itself disposed external to the cell. Such cell surface expressed integrins include for example VLA-4 ( $\alpha_4\beta_1$ ) and VLA-5 ( $\alpha_5\beta_1$ ).

30 N. L. Kovack, *et al.* in J. Cell Biol. 116:499-509, 1992 discloses that cell surface expressed integrins can be activated by an anti-integrin antibody so that the activated cell surface expressed integrin will aid in cellular adhesion to a variety of substrates.

As used herein, the term "anti-integrin antibody" refers to an antibody capable of binding to an integrin. According to the present invention, the anti-integrin antibody can include, by way of example and without limitation, the monoclonal antibodies (Mabs) anti- $\beta_1$ , , 8A2, TS2/16.2.1, anti- $\beta_2$ , anti- $\beta_3$ , and anti- $\beta_1$ , as well as polyclonals.

The cell surface expressed integrins may also be activated by other methods including antibodies, cytokines, divalent cations and peptides.

When practicing one of the immobilization methods of the invention, an appropriate combination of cell surface expressed adhesion molecule activators and adhesive should be selected. Integrin and adhesive combinations known to bind are shown in Table 1.

Table 1

$\beta$ subunit	$\alpha$ subunit	Adhesive
$\beta_1$	$\alpha_1$	collagens, laminin
	$\alpha_2$	collagens, laminin
	$\alpha_3$	laminin, fibronectin, epiligrin, collagen
	$\alpha_4$	VCAM-1, fibronectin
	$\alpha_5$	fibronectin
	$\alpha_6$	laminin
	$\alpha_7$	laminin
	$\alpha_v$	vitronectin, fibronectin
$\beta_2$	$\alpha_L$	ICAM-1, ICAM-2, ICAM-3
	$\alpha_v$	iC3b, IAM-1, fibrinogen, factor X
	$\alpha_X$	fibrinogen, iC3b
$\beta_3$	$\alpha_{IIB}$	fibrinogen, fibronectin, vWF, vitronectin
	$\alpha_v$	thrombospondin vitronectin, fibrinogen, fibronectin VWF, thrombospondin, osteospondin
$\beta_4$	$\alpha_6$	laminin, basement membrane protein
$\beta_5$	$\alpha_v$	vitronectin
$\beta_6$	$\alpha_v$	fibronectin
$\beta_7$	$\alpha_4$	VCAM-1, fibronectin, MAdCAM-1

As discussed above, non-adherent cells can be attached to a surface via antigen-specific Mabs, which themselves are directly attached (i.e., covalently bound) to the surface. Such antigen-specific Mabs include, for example, anti-CD34, anti-CD4, and anti VLA-4, anti-VLA-5.

The method of detachment of immobilized cells from a surface will be selected according to the immobilization method employed. When the method of cell surface expressed integrin mediated immobilization onto an immobilization surface coated with an adhesive is employed, cellular detachment is accomplished by competition with peptide(s) (e.g., from fibronectin), release by protease treatment (e.g., trypsin), release with cell disassociation buffer, polyanions which may interfere with the heparin binding site, cations such as  $\text{Ca}^{++}$  which interfere with integrin function, or simple disruption by pipetting. When a cell is immobilized by binding to an antibody which is directly, i.e., covalently, bound to an immobilization surface, such as with glutaraldehyde, cell detachment is accomplished by either the methods mentioned above or competition by excess molecule to which the antibody binds, or excess antibody or antibody fragments (such as Fabs).

When practicing another embodiment of the non-adherent cell immobilization method of the invention, a holding pipette is employed. As used herein, the term "holding pipette" refers to a microcapillary having an inner bore diameter (opening) of about 0.5 microns to about 2.5 microns which is capable of holding a non-adherent cell without puncturing or otherwise damaging the non-adherent cell surface. The bore of the holding pipette is under a reduced pressure (vacuum) which provides the force by which a non-adherent cell is drawn to and immobilized onto a distal end of the holding pipette. The holding pipette of the invention is made according to Example 13. The holding pipette can be made using a DeFonbrune microforge. Appropriate bends are made in a glass capillary so that it fits in a chuck assembly holder such that an about 1 to about 5 gram weight can be hung from the capillary positioned inside a heating filament. Heat is applied softening the glass resulting in the weight pulling the glass capillary to an about 1 to an about 3 micron diameter at which point the piece of glass capillary from which the weight is suspended breaks away leaving a holding pipette with an about 1 to an about 3 micron diameter tip. The tip is then brought close to the heating filament and the tip is heat

polished resulting in a smooth tip with an opening of about 0.5 to about 2.5 microns. This holding pipette can then be attached to a syringe assembly that can be used to create a vacuum that will hold the cell in place during the microinjection procedure.

When making such a holding pipette, one is not limited to the DeFonbrune microforge. For example, any of the commercially available needle pullers (Sutter  
5 Instruments, Narishige, Kopf) can be used to pull a capillary to the above dimensions. However, the tip must still be heat polished with a microforge (DeFonbrune or Narishige).

Generally, the holding pipette used in the invention will have an O.D. less than about 2.5 microns such that a non-adherent cell held by the holding pipette will not be  
10 aspirated into the pipette. In order to minimize penetration of the cell wall by the holding pipette, the holding pipette tip will have to be fire-polished or suitably smoothed. Either the Eppendorf or Zandler syringe assemblies have been designed to produce a vacuum in the holding pipette that will hold the cell in place during the injection procedure, but not rupture the membrane of the cell in the process.

Barbs should be removed from the holding pipette tip. This is generally accomplished by fire-polishing. Care should be taken not to seal the holding pipette during  
15 fire-polishing. This can be accomplished by passing a gentle stream of air (or an inert gas) through the holding pipette while heating the tip to about .5 to about 2.5 microns, which is sufficient to anneal but not melt the holding pipette tip.

To facilitate monitoring of cell viability post-microinjection, non-adherent cells micro-injected with foreign material such as DNA can be co-micro-injected with either  
20 fluorochrome- (e.g., FITC, Oregon green, Rhodamine) coupled dextran (MW of various molecular weights, e.g., 150,000), or a vital fluorescent DNA-stain (e.g., Hoechst 33342 yoyo dye), Green Fluorescent Protein (GFP), or DNA encoding the GFP reporter gene.  
25 Depending upon cell culture conditions used pre- and post-microinjection, micro-injected non-adherent cells can be driven into cycle with hematopoietic cytokines or growth factors such as IL-3, IL-6, SCF and Flt-3 ligand or with other agents, such as neutralizing anti-TGF- $\beta$  antibody. Alternatively, it may be preferable to maintain cells in culture conditions which enable survival without inducing cycling or differentiation. It should be noted that  
30 post-microinjection, non-adherent cells that have been detached from the immobilization surface will regain their non-adherent properties and can be grown again in suspension as non-adherent cells.

The microinjection method and apparatus of the invention are described in Example 1.

A stock solution containing foreign material, indicated as "Sample" in Table 2, was typically buffered.

5 A stock solution of foreign material, indicated as "Sample" in Table 2, typically included phosphate-buffered saline (PBS), water and Tris-EDTA (TE). The stock solution was then either directly micro-injected into the recipient cells or diluted with a second buffer solution prior to microinjection into the recipient cells. The second buffer solution typically contained Hepes (50 mM), KCl (100 mM) and  $\text{NaH}_2\text{PO}_4$  (5 mM) and had a pH  
10 of about 7.2. The concentration, in mg/ml, of foreign material ("Sample") actually micro-injected into the cells is indicated as "Conc." in Table 2. The sample solution was typically centrifuged at about 10,000 to 15,000 rpm using a table-top Eppendorf micro-centrifuge, or filtered through a 0.02 mm or 0.1 mm membrane and/or dialyzed prior to microinjection into the cells.

15 The cells receiving the sample solution by microinjection included primary cells or transformed cells (e.g., U937, TF-1 cells) ( $\text{CD34}^+$ ,  $\text{CD34}^+/\text{CD38}^-$ ,  $\text{CD34}^+/\text{CD38}^+$ , and  $\text{CD34}^+/\text{CD38}^+/\text{Thy-1}^{\text{lo}}$ ).  $\text{CD34}^+$ -expressing cells are primary human stem/progenitor cells immunomagnetically purified from umbilical cord blood. The  $\text{CD34}^+/\text{CD38}^-$  and  $\text{CD34}^+/\text{CD38}^+/\text{Thy-1}^{\text{lo}}$  cell populations were isolated by fluorescent activated cell sorting (FACS).  
20 The  $\text{CD38}^-$  subpopulation of the  $\text{CD34}^+$  cells comprises a more primitive subset of cells. 3T3 denotes the Swiss 3T3 fibroblast cell line, which is an adherent mouse fibroblast cell line. U937 is a generally non-adherent human myelomonocytic cell line.

The sample foreign material micro-injected into the cells included DNAs (pCMV- $\beta$ , ph-GFP, pGreen Lantern ("pGreen"), pCMV- $\beta$ /ph-GFP in a 1:1 concentration  
25 ratio), fluorescent conjugates (PE- $\beta$ -RAM, FITC-GAM, rhodamine-dextran, FITC-dextran), or mixtures thereof. Various combinations of these and other foreign materials previously described can be micro-injected in the indicated cell lines.

The cells were immobilized onto a surface, typically a tissue culture plate or Petri dish, by a variety of methods by treating the surface with a molecule "adhesive" (e.g.,  
30 fibronectin, "Fn") (Table 2). In some cases, "activators" (e.g., anti- $\beta_1$ , integrin monoclonal antibodies) 8A2 Ab (8A2 from N. Kovach) and TS2 sup (conditioned supernatant from the TS2/16.2.1 hybridoma cell line, T. Springer, ATCC no. HB-243) are anti- $\beta_1$ , integrin

monoclonal antibodies which were used for activating cell surface-expressed integrins on respective cells for their immobilization onto fibronectin-coated plates. Con A (concanavalin A) was used to immobilize respective cells by first treating the immobilization surface with glutaraldehyde and then exposing the derivatized surface first to concanavalin A and then to respective cells. Biotin CD34 immobilization was attempted by exposing glutaraldehyde derivatized plates to biotinylated anti-CD34 antibody and respective cells.

The number of cells plated in the cloning ring prior to microinjection generally ranged from about 300 to about 2000. Only a fraction of these were actually micro-injected. The number of cells micro-injected per experiment, indicated as "Cells (#)" in Table 3, ranged from about 2 to about 150 and the microinjections were identified as "good" (g) or "not acceptable" (na). "Good" microinjections provided cells: (1) that showed a mild swelling of the nucleus during microinjection; (2) which volume of sample solution received was not so great as to destroy the cell; and (3) that were not immediately destroyed by the penetration or retraction of the microinjection needle. Microinjections failing any of these criteria were identified as "not acceptable".

The cells were assayed or visually monitored immediately after microinjection and at four hours, 12-18 hours, 48 hours and greater than 72 hours after microinjection. The number, size, viability status and/or color and intensity of fluorescence of the micro-injected cells was monitored by phase contrast and fluorescent microscopy. Red fluorescence was from rhodamine micro-injected into the cell and green fluorescence was from FITC-dextran or GFP expression by the micro-injected cell. Occasionally, the expression of beta-gal by the micro-injected cell was monitored by x-gal staining.

Although the microinjections were generally conducted on a heated (about 37°C) microscope stage, some were conducted at ambient temperature (about 20°-24°C).

Following immobilization of the primary cells or transformed cultures onto the immobilization surface coated with an adhesive, aliquots of stock solutions containing varying concentrations of foreign material were micro-injected into the immobilized cells. Microinjection needles having an O.D. in the ranges of about 0.9 to about 1.1 microns (indicated as Eppendorf "Femtotips"), about 0.45 to about 0.60 microns (indicated as "fine"), or specific values (as indicated in Table 2) were prepared and employed. The results are summarized in Tables 2 and 3.

TABLE 2

EXP. #	SAMPLE (DNA, protein, dextran)		CELLS FOR INJECTION	Adhesive	Activator	NEEDLE O.D.
	Sample	conc. (mg/ml)	cell			
1	pCMV- $\beta$	0.2	CD34 <sup>+</sup> CD38 <sup>-</sup>	Fn	8A2 Ab	fine
2	pCMV- $\beta$	0.2	CD34 <sup>+</sup> CD38 <sup>+</sup>	Fn	8A2 Ab	Femtotips
5	FITC GAM	stock	CD34 <sup>+</sup>	Fn	8A2 Ab	fine
20	pGreen:pCMVb(1:1)	0.25:0.1	CD34 <sup>+</sup>	Fn	8A2 Ab	fine
21	pGreen:pCMVb(1:1)	0.25:0.1	CD34 <sup>+</sup>	Fn	8A2 Ab	fine
22	pGreen:pCMVb(1:1)	0.25:0.1	CD34 <sup>+</sup>	Fn	8A2 Ab	fine
23	pGreen:pCMVb(1:1)	0.25:0.1	CD34 <sup>+</sup>	Fn	8A2 Ab	fine

Exp. #	SAMPLE (DNA, protein, dextran)		CELLS FOR INJECTION			NEEDLE O.D.
84	FITC-Dextran	2.5-filtered	SD34 <sup>+</sup> CD38 <sup>+</sup> Thy1 <sup>+</sup>	Fn	TS2sup	0.2μ
85	FITC-Dextran	2.5-filtered	CD34 <sup>+</sup>	Fn	TS2sup	0.2μ
92	FITC-Dextran	2.5-filtered	CD34 <sup>+</sup> CD38 <sup>+</sup> Thy1 <sup>+</sup>	Fn	TS2sup	0.2μ
93	pGreen	0.1-spun	CD34 <sup>+</sup> CD38 <sup>+</sup> Thy1 <sup>+</sup>	Fn	TS2sup	0.2μ
99	FITC-Dextran	2.5-filtered	CD34 <sup>+</sup>	con A	None	0.2μ
100	FITC-Dextran	2.5-filtered	CD34 <sup>+</sup>	Biotin/ CD34 CD34	None	0.2μ
101	FITC-Dextran	2.5-filtered	CD34 <sup>+</sup>	Fn	TS2sup	0.2μ
102	FITC-Dextran	2.5-filtered	CD34 <sup>+</sup>	Fn	TS2sup	0.2μ



EXP. #	SAMPLE (DNA, protein, dextran)		CELLS FOR INJECTION			NEEDLE O.D.
103	FITC-Dextran	2.5-filtered	CD34 <sup>+</sup>	con A	None	0.2 $\mu$
104	FITC-Dextran	2.5-filtered	CD34 <sup>+</sup>	con A	None	0.2 $\mu$
113	FITC-Dextran	2.5-spun	CD34 <sup>+</sup>	Fn	TS2sup	0.2 $\mu$
114	FITC-Dextran	2.5-spun	CD34 <sup>+</sup>	Fn	TS2sup	0.2 $\mu$
115	FITC-Dextran	5.0-spun	CD34 <sup>+</sup> /CD38 <sup>+</sup>	Fn	TS2sup	0.2 $\mu$

TABLE 3

EXP. #	# of injected cells	# OF POSITIVE CELLS (E.G., FLUORESCENT)				HEATED STAGE
		<4 hrs	4 hours	O/N	>2 d	
1	40	ND	ND	ND	0(5 days) X-gal	no
2	7	ND	ND	ND	0(5 days) X-gal	no
5	20	4 (immed.)	0	ND		no
20	50(37g/13na)	ND	0	0		no
21	79(61g/18na)	ND	2	3		no
22	53(45g/8na)	ND	3	2	3(3 days)	no
23	108(62g/46na)	ND	5	4	X-gal	no
84	32g	9	ND	3	1	yes
85	60g	35-40	ND	19	16	yes
92	50g	27	27	26	16	yes
93	70g	ND	8	5	0	yes
99	20g	7	4	ND		yes
100	20g	5	4	ND		yes
101	25g	16	12	ND	NA	yes
102	25g	7	3	ND	NA	yes
103	30g	6	2	ND	NA	yes
104	20g	5	1	ND	NA	yes
113	100g	50	ND	ND	NA	yes
114	50g	3	ND	ND	NA	yes
115	50g	10	ND	ND	NA	yes

The results indicate that non-adherent cells and, in particular, primary primitive hematopoietic cells from the CD34<sup>+</sup> stem/progenitor population, can be successfully micro-injected with various macromolecules including DNA by employing the novel microinjection method and apparatus of the invention. The genetically modified cells successfully express the respective product of the genetic foreign material micro-injected into them.

In some embodiments of the invention, a sufficient amount (generally less than 5-10% by volume of the cell nucleus being micro-injected) of a stock solution containing a nucleic acid foreign material is micro-injected into a non-adherent cell, more preferably a hematopoietic stem cell or other hematopoietic cell, immobilized onto an immobilization surface by way of an adhesive and possibly an activating agent that activates cell surface-expressed integrins on the non-adherent cell. The microinjection is accomplished by employing a microinjection needle having an outer diameter less than about 0.20 microns and in some embodiments between about 0.05 to about 0.15 microns.

## 15 Gene Therapy

The present invention also provides a method for the transduction of hematopoietic stem cells (hSCs) and thus an alternative strategy for their direct genetic modification by: (1) direct delivery of DNA sequences into the nuclei of hSCs by microinjection; (2) integration of micro-injected transgene sequences in the chromatin of hSCs or extrachromosomal maintenance of the transgene sequences on episomal vectors or artificial human chromosomes and persistence of those sequences in the progeny of said hSCs; and (3) microinjection of sufficiently large (15-25 Kb) transgenic DNA constructs containing regulatory elements, such as promoters, enhancers and locus control regions (LCRs), and intron/exon structure necessary for appropriate long-term, cell type-specific expression of the introduced transgenes; and 4) microinjection of DNA/protein mixtures with the protein(s) included in the injection sample which aid in gene integration and/or targeting (Figs. 8 & 9).

Genetically modified hSCs prepared according to the methods of the present invention can be employed for gene therapy applications once said modified hSCs have been delivered to humans for long-term reconstitution.

According to the present invention, hematopoietic stem cells that have been modified by microinjection of foreign material can be used to treat a variety of physiological disorders

such as, by way of example and without limitation, AIDS, cancer, thalassemia, anemia, sickle cell anemia, adenosine deaminase deficiency, Fanconi Anemia, Gaucher disease, Hurler Syndrome, immune deficiencies, and metabolic diseases.

The physiological disorders contemplated within the invention will be responsive to gene therapy. By "responsive to gene therapy" is meant that a patient suffering from such disorder will enjoy a therapeutic or clinical benefit such as improved symptomatology or prognosis.

As indicated above, one aspect of the present invention relates to the use of modified hSCs, as cellular vehicles for gene transfer. The genes, or transgenes, can be any gene having clinical usefulness, for example, therapeutic or marker genes or genes correcting gene defects (e.g., mutant hemoglobin genes in thalassemia or sickle cell anemia) in blood cells. Preferably, the primary human cells are blood cells. The term "blood cells" as used herein is meant to include all forms of blood cells as well as progenitors and precursors thereof, as hereinabove described.

Thus, in one embodiment, the invention is directed to a method of enhancing the therapeutic effects of hSCs, comprising: (i) micro-injecting into the hSCs of a patient a DNA segment encoding a product that enhances the therapeutic effects of the human primary cells; and (ii) introducing the genetically modified hSCs into the patient.

The DNA produces the agent in the patient's body and, in accordance with such embodiment, the agent is expressed at the tissue site itself. Similarly, as hereinabove indicated, hSCs which are genetically engineered need not be targeted to a specific site and, in accordance with the invention, such engineered hSCs and their progeny function as a systemic therapeutic; e.g., a desired therapeutic agent can be expressed and secreted from the cells systemically.

More specifically, there is provided a method of enhancing the therapeutic effects of hSCs that are infused in a patient, comprising: (i) micro-injecting into the hSCs of a patient a DNA segment encoding a product that enhances the therapeutic effects of the blood cells; and (ii) introducing cells resulting from step (i) into the patient.

When the modified hSCs are not "targeted," the genes are inserted in such a manner that the patient's transformed blood cells which are progeny of the modified hSCs will produce the agent in the patient's body (Fig. 7).

The primary human blood cells that are the progeny of modified hSCs and which can be used in the present invention include, by way of example, leukocytes, granulocytes, monocytes, macrophages, lymphocytes, and erythroblasts. For example, stem-cells from thalassemic or sickle cell anemia patients that are genetically modified with the appropriate  
5 hemoglobin gene may give rise to genetically corrected red blood cells.

The DNA carried by the hSCs can be any DNA having clinical usefulness, for example, any DNA that directly or indirectly enhances the therapeutic effects of the cells. Alternatively, the DNA carried by the hSCs can be any DNA that allows the hSCs to exert a therapeutic effect that the hSCs would not normally exert. Examples of suitable DNA that  
10 can be used for genetically engineering, for example, blood cells, include those that encode cytokines such as tumor necrosis factor (TNF), interleukins (for example, interleukins 1-12), globin genes, DNA-repair genes, drug-resistance genes, Fanconi Anemia genes and anti-HIV (Human Immunodeficiency Virus) resistance genes.

The DNA which is used for transducing the human cells can be one whose expression  
15 product is secreted from the cells. Alternatively, it may encode for gene products retained within the cell. The human cells can also be genetically engineered with DNA which functions as a marker, as hereinafter described in more detail.

In one aspect, the inserted genes are marker genes which permit determination of the traffic and survival of the transformed cells *in vivo*. Examples of such marker genes include  
20 the neomycin resistance (neoR) gene, multi-drug resistant gene, thymidine kinase gene,  $\beta$ -galactosidase, dihydrofolate reductase (DHFR) and chloramphenicol acetyl transferase.

The hSCs are genetically engineered *in vitro*. For example, cells may be removed from a patient and stem cells isolated; genetically engineered *in vitro* with DNA encoding the therapeutic agent, with such genetically engineered hSCs being readministered along with  
25 a pharmaceutically acceptable carrier to the patient. Such a treating procedure is sometimes referred to as an *ex vivo* treatment.

In some embodiments, the progeny of the modified hSCs are primary human cells and more preferably are primary human nucleated blood cells which express in the appropriated progeny cells the product of the genetic foreign material micro-injected into the parent hSCs.

30 The pharmaceutically acceptable carrier may be a liquid carrier (for example, a saline solution) or a solid carrier; for example, an implant of a biocompatible and non-immunogenic material. In employing a liquid carrier, the engineered cells may be introduced parenterally,

e.g., intravenously, sub-cutaneously, intramuscularly, intraperitoneally, intralesionally, or directly into the bone marrow.

Results from mouse, large animal, and human studies permit a reasonable estimate of the number of stem cells that need to be delivered to humans for long term reconstitution.

- 5 Since the genetic therapies under consideration will frequently be directed to children, estimates herein are based on their smaller body weight. Furthermore, although a significant number of unmarked, short-term reconstituting cells may need to be co-delivered to ensure rapid engraftment and survival, our focus is on the much smaller number of gene-modified, long-term reconstituting stem cells.

- 10 Three independent mouse studies have reported long-term reconstitution with as few as about 100 marrow cells (Spangrude *et al.*, 1988), 10 marrow cells (Jones *et al.*, 1996), or even 1 marrow cell (20% of mice reconstituting (Osawa, *et al.*, 1996). If direct scaling by weight alone is appropriate, an average reconstitution requirement of about 5 cells for mice would extrapolate into approximately about a 5,000 marrow cell requirement for a human  
15 child. Whether one needs to also scale for the increased human life span is not yet clear.

- In human marrow transplantation, the minimal dose typically delivered is  $1 \times 10^8$  nucleated cells per kg body weight, equivalent to  $2.5 \times 10^9$  cells for a 25 kg child. Reported experimental data and modeling of feline hematopoiesis indicate that the stem cell frequency is approximately 1 in  $1.7 \times 10^6$  marrow cells (Abkowitz *et al.*, 1996). If this same frequency  
20 holds for human marrow, delivery of  $2.5 \times 10^9$  cells corresponds to delivery of 1450 stem cells.

- Children reportedly reconstitute with as little as 30 mls of transplanted cord blood, likely due to the significant proliferative potential of primitive hematopoietic cord blood cells (Kurtzberg *et al.*, 1996). Assuming approximately  $1.5\text{--}3 \times 10^8$  nucleated cells in this volume,  
25 with a stem cell frequency of 1 in  $10^5$  to  $10^6$  this translates into successful engraftment with as few as 150-3,000 stem cells.

- Reported evidence from engraftment of human cord blood cells in NOD/SCID mice suggests a very close relationship between NOD/SCID reconstituting cells (SRCs) and human stem cells. SRCs are present at a frequency of  $\sim 1$  in  $10^4$  CD34<sup>+</sup> cells (Serrano *et al.*,  
30 1996). Thus, 30 mls of cord blood (with approximately  $3 \times 10^7$  mononuclear cells, 1% of which are CD34<sup>+</sup>) contain approximately 30 SRCs. Even if the seeding efficiency of SRCs in NOD/SCID mice is only 10%, this translates into 300 SRCs.

Thus, all four calculations suggest the required number of stem cells may be in the range of about 150 to about 5,000. Since successful reconstitution depends not only on stem cell delivery, but rapid engraftment by short-term progenitors, it is possible that the stem cell requirements are even less than those calculated above.

- 5 Clinical benefit for genetic diseases will generally require correction in a significant fraction of the stem cells present *in vivo*. This may be accomplished by *in vitro* selection for modified hSCs prior to engraftment (so that only the successfully transduced cells are transplanted into the patient) and/or by subsequent *in vivo* selection for modified hSCs (to enrich for modified hSCs at the expense of endogenous, unmodified hSCs). This may require
- 10 transduction of hSCs with two independently regulated genes present on the same DNA construct: the selectable gene targeted for expression in stem/progenitor cells and the therapeutic gene (e.g., ADA or globin) targeted for expression in the required cell type.

- Transduction of hSCs with the human O<sup>6</sup>-methylguanine DNA methyltransferase (MGMT) gene may enable *in vivo* selection of surviving, modified hSCs by briefly treating
- 15 patients with alkylating agents of the nitrosourea class (e.g., 1,3-bis (2-chloroethyl)-1-nitrosourea; BCNU). Whereas most anti-neoplastic drugs (e.g., Taxol) are toxic to cycling hematopoietic progenitors, sparing the quiescent hematopoietic stem cells, nitrosoureas such as BCNU also exert their DNA-damaging and toxic effects directly on the stem cells. MGMT, which reportedly removes O<sup>6</sup>-alkylguanine induced in DNA by various alkylating
- 20 agents (Mitra *et al.*, 1993), is reportedly normally expressed at very low levels in hematopoietic stem/progenitor cells (Wang *et al.*, 1996; Moritz *et al.*, 1995). However, when exogenously expressed in cells, MGMT reportedly confers cell resistance to BCNU, CCNU, dacarbazine, N-methyl-N'-nitro-N'-nitrosoguanidine, temozolomide, and streptozotocin (Preuss *et al.*, 1996; Spain *et al.*, 1992). For example, mice expressing MGMT in their stem
- 25 cells were reportedly resistant to BCNU-induced hematosuppression (Maze *et al.*, 1996). Although the human multiple drug resistance gene (MDR-1) has been proposed for *in vivo* selection of transduced stem cells, the fact that human stem cells already reportedly constitutively express MDR-1 (Chaudhary *et al.*, 1991) suggests that any enrichment for transduced cells by MDR-1 resistant drugs (e.g., taxol) may occur at the level of progenitors
- 30 but not stem cells. As is true for any proposed *in vivo* selection (e.g., CCNU, taxol) for marked hematopoietic cells, it will be imperative to minimize drug toxicity for other organs and cells. Finally, MGMT transgene expression, by itself, should, as previously reported,

confer resistance in hematopoietic cells to agents such as BCNU employed in high-dose or repetitive chemotherapy for breast and other cancers (Chabner *et al.*, 1993).

In one aspect, gene therapy employing genetically modified hematopoietic stem cells may include the following elements. Approximately  $1-10 \times 10^3$  highly enriched  
 5 hematopoietic stem cells are obtained from human cord blood and are temporarily immobilized. Microinjection of these cells delivers a reproducible volume-containing DNA and possible integration enzyme(s) - such that 1-3 copies of the DNA are successfully integrated per cell. Microinjected DNAs of 15-25 kb in size, containing two independently regulated transgenes, are integrated without arrangement. One transgene, targeted for  
 10 expression in stem cells, provides for *in vitro* (e.g., rsGFP, or truncated nerve growth factor receptor; tNGF-R) or *in vivo* (e.g., MGMT) selection of transduced stem cells. The therapeutic transgene (e.g., ADA for ADA SCID, globin for hemoglobinopathies, MDR-1 for chemoresistance) is targeted for expression in the appropriate hematopoietic cells.

The present invention may be employed for introducing relatively large fragments of  
 15 nucleic acid into a cell. For example, nucleic acid sequences of DNA having molecular weights of between 20 kb and 24 kb have been introduced through an opening of 0.1 microns. Hence, it is anticipated that the microneedles of the present invention having an opening of 0.1 microns may be used with such relatively large molecules.

The relatively large-sized DNA can be filtered through a 0.1 micron filter. In  
 20 addition, the present studies demonstrate that the passage of these relatively large molecules was achieved without loss of integrity of the molecule (Fig. 11).

The following abbreviations have been used in the preparation of this disclosure.

25	pCMV- $\beta$	DNA plasmid expressing the $\beta$ -gal reporter gene under control of the cytomegalovirus (CMV) promoter/enhancer sequences.
	PE-RAM	Phycoerythrin (a fluorochrome) labeled rabbit anti-mouse Immunoglobulins
	FITC GAM	Fluorescein Isothiocyanate (a fluorochrome) labeled goat anti-mouse Immunoglobulins



	phGFP	DNA plasmid expressing the humanized red shifted green fluorescent protein (GFP) reporter gene under control of the CMV promoter/enhancer sequences (from ClonTech)
	pGreen	DNA plasmid (pGreen Lantern) expressing the humanized red shifted GFP reporter gene under control of the CMV promoter/enhancer sequences (from GIBCO-BRL)
5	rhodamine-Dextran	Rhodamine (a fluorochrome) coupled to dextran
	FITC-Dextran	FITC coupled to dextran
	CD34+CD38-Thy-1+	the CD38 <sup>-</sup> Thy-1 <sup>+</sup> (actually Thy-1 <sup>+</sup> ) subpopulation of CD34 <sup>+</sup> cells isolated by FACS.
10	3T3	Swiss 3T3 fibroblasts-an adherent mouse fibroblast cell line
	U937	a human myelomonocytic cell line, normally non-adherent.
15	8A2 Ab and TS2 sup	anti- $\beta_1$ integrin-mediated attachment to fibronectin-coated plates. 8A2 (N. Kovach) and TS2/16.2.1 (T. Springer, ATCC) are murine monoclonal antibodies specific for human $\beta_1$ integrin.
20	hSC	hematopoietic stem cell

Materials used herein were obtained as follows: PE-RAM (B-D, Beckton Dickinson), phGFP (ClonTech), pGreen (Gibco), rhodamine-dextran (Molecular Probes), FITC-Dextran (Sigma Chemical Co. and Molecular Probes). pCMV- $\beta$  and FITC GAM were prepared as described in the present disclosure, and are available in the inventor's laboratory. Some materials were obtained from Sigma Chemical Company. Cell types described herein were obtained from the American Type Culture Collection.

The foregoing will be better understood with reference to the following examples which detail certain procedures according to the present invention. All references made to

these examples are for the purposes of illustration. They are not to be considered limiting as to the scope and nature of the present invention.

#### EXAMPLE 1

##### DELIVERY OF FITC-DEXTRAN TO IMMOBILIZED CD34<sup>+</sup> CELLS

The present example demonstrates the utility of the invention for introducing a molecule of relatively small size into a living cell, wherein the cell retains viability and is provided in an immobilized state during the time the molecule is being introduced into the cell.

##### Purification and Culturing of CD34<sup>+</sup> Cells

The CD34<sup>+</sup> antigen, present on approximately 0.5-1.0% of mononuclear bone marrow and umbilical cord blood cells, marks all measurable human hematopoietic stem and progenitor cells (Fig. 5). Umbilical cord blood cells were obtained from normal human fetal deliveries, and mononuclear cells were purified by centrifugation over Ficoll-hypaque. CD34<sup>+</sup> cells were isolated by immunomagnetic selection with the Miltenyi MiniMACS CD34 Multisort Isolation Kit (involves (1) incubation of cells with anti-CD34 antibody coupled via dextran to immunomagnetic particles, (2) isolation of magnetically-labeled cells by passing through a column attached to a magnet, (3) release of cells from magnetic particles by cleavages with dextranase, (4) separation of cells from magnetic particles by passing through column attached to a magnet). Subsequent FACS analysis, with another anti-CD34 antibody recognizing a different CD34 epitope, demonstrated that the cells were 85-95% pure for CD34 expressing cells. Purified cells were maintained overnight (18 hrs) in serum free medium (Iscoves Modified Dulbecco's Medium (IMDM, Gibco) supplemented with bovine serum albumin (2%, StemCell Technology), insulin (10 micrograms/ml), transferrin (200 microgram/ml, ICN), 2-mercaptoethanol (0.05 mM, Sigma), low-density lipoprotein (40 microgram/ml, Sigma), and pen-strep (100 units and 50 microgram/ml, respectively) containing 20 ng/ml human Flt-3 ligand (Peprotech), 20 ng/ml human Interleukin-3 (IL-3, Peprotech), and 20 ng/ml human Stem Cell Factor (SCF, Peprotech) [IMDM/F-3-S] at 37°C with 6% CO<sub>2</sub>.

#### Preparation of Fibronectin-Coated Surface

A 6 mm glass cloning ring was attached via Vaseline(R) to a 35 mm tissue culture dish (Corning). The dish surface enclosed by the cloning ring was coated with fibronectin by adding 30-50 microliters of a 50 microgram/ml fibronectin solution (Boehringer Mannheim, #1051-407) in phosphate buffered saline (PBS, Sigma), and incubating overnight at 4 °C (alternatively, can be for 45 min. at room temperature). Excess fibronectin-containing solution was removed from the cloning ring immediately prior to addition of cells.

#### Attachment of CD34<sup>+</sup> Cells to Fibronectin-Coated Dish

After overnight culture, cells were prepared at a concentration of  $8 \times 10^4$  cells/ml in IMDM/F-3-S. This cell-containing media (25 microliters containing approximately 2000 cells) was mixed with 25 microliters of media (IMDM) conditioned 2 days by the TS2/16.2.1 hybridoma cell line (ATCC #HB-243 which produces antibody reactive with Integrin  $\beta_1$ -human CD29). The 50 microliters of cell/antibody mixture was placed into a 6 mm glass cloning ring enclosing the fibronectin-coated surface. Cells, in the presence of antibody, were allowed to attach to fibronectin for greater than 30 min. at 37 °C in the presence of 6% CO<sub>2</sub>. Figure 10A shows an example of cells incubated on a fibronectin surface without the addition of the activating TS2/16.2.1 antibody. Cells are loosely attached and will not withstand microinjection. Figure 10B shows an example of cells incubated on a fibronectin surface in the presence of the activating TS2/16.2.1 antibody. The cells are more spread with the presence of numerous microspikes and will withstand the microinjection process. Subsequently, 1 ml of IMDM/F-3-S was added outside the cloning ring, and the 35 mm plate containing cells and cloning ring was spun at 600 rpm for 5 min (Beckman low-speed GS-6R centrifuge, swinging bucket rotor, brake off).

#### Microinjection of CD34<sup>+</sup> Cells with FITC-Dextran

Fine glass microinjection needles were prepared from thin-walled borosilicate glass capillaries (Sutter, 1.2 mm O.D., 0.94 mm I.D.) with an automated pipette puller (Sutter, P-87, 3 mm box filament). Scanning Electron Microscopy (SEM) was used to determine the outer diameter of microinjection needles pulled with the identical program; O.D.s between 0.17 and 0.22 micron were obtained. FITC-dextran (150,000 M.W., Sigma) at a concentration of 0.25% (weight per volume) in 50 mM Hepes (pH 7.2/100 mM KCl/5 mM

MISSING UPON TIME OF PUBLICATION

EXAMPLE 2  
DELIVERY OF FITC-DEXTRAN TO  
IMMOBILIZED CD34<sup>+</sup>/CD38<sup>-</sup>/THY-1<sup>lo</sup> PRIMITIVE CELLS

5           The present example demonstrates the utility of the present invention for the stable incorporation of a foreign molecule into a cell, and particularly immature, undifferentiated cells.

Purification of Primitive CD34<sup>+</sup>/CD38<sup>-</sup>/Thy-1<sup>lo</sup> Cells

10           CD34<sup>+</sup>/CD38<sup>-</sup>/Thy-1<sup>lo</sup> cells comprise approximately 1-4% of CD34<sup>+</sup> cells, and exhibit properties consistent with that of highly primitive hematopoietic cells (highly enriched in long term culture initiating cells (LTC-ICs)). As such, they represent a candidate population of stem cells. These cells were purified by first immunomagnetically isolating CD34<sup>+</sup> cells (Miltenyi Minimacs, see Example 1) followed by fluorescence activated cell sorting (FACS)  
15           with PerCP-CD34 (Becton-Dickinson), PE-CD38 (Becton-Dickinson), and FITC-Thy-1 (Pharmingen) antibodies. Cells were sorted with Becton-Dickinson FACS Vantage with automated cell deposition unit. The primitive nature of these cells was further confirmed by the vast majority of them expressing the CD45Ra<sup>+</sup>/CD71<sup>-</sup> phenotype.

          Culturing of cells, attachment of fibronectin-coated dishes, microinjection of cells  
20           with FITC-dextran, monitoring and subsequent culture were performed as described in Example 1. Cells were well attached to the fibronectin, in that they were not dislodged during microinjection. Of 32 CD34<sup>+</sup>/CD38<sup>-</sup>/Thy-1<sup>lo</sup> cells (Experiment 84, Tables 2-3; Fig. 4), each micro-injected with an estimated 2-10 femtoliters of 0.25% FITC-dextran, 9 cells were positive for fluorescence 30 min. post-microinjection. Three fluorescent cells were still  
25           present 24 hrs. post-microinjection.

EXAMPLE 3  
EXPRESSION OF RED-SHIFTED GREEN FLUORESCENT  
PROTEIN BY MICROINJECTED, IMMOBILIZED CD34<sup>+</sup> CELLS

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          The present example demonstrates the utility of the present invention for the stable incorporation of a foreign nucleic acid sequence encoding a protein into a cell, and the successful expression of that protein by the modified cell. In doing same, the present example also demonstrates the utility of the present invention as a gene therapy technique.

Using 0.45 micron microinjection needles, Green Fluorescent Protein (GFP) reporter gene expression, 24-48 hrs. post-microinjection, in 5-15% of immobilized CD34<sup>+</sup> cells (6-8 micron diameter) was obtained. Cells were micro-injected with approximately 20-40 femtoliters of 50 ng/microliter pGreen Lantern DNA plasmid (Gibco BRL) which expresses the humanized red shifted Green Fluorescent Protein under control of the cytomegalovirus (CMV) promoter/enhancer. The remainder of the cells were killed by microinjection-pipette induced cell damage or delivery of too much volume.

By employing microinjection needles having an outer diameter of about 0.2 microns, GFP expression 24-48 hrs. post-injection in 5%-15% of immobilized CD34<sup>+</sup> cells was obtained.

#### EXAMPLE 4

##### EXPRESSION OF RED-SHIFTED GREEN FLUORESCENT PROTEIN BY MICROINJECTED IMMOBILIZED CD34<sup>+</sup>/CD38<sup>+</sup>/Thy-1<sup>lo</sup> CELLS

CD34<sup>+</sup>/CD38<sup>+</sup>/Thy-1<sup>lo</sup> cells were isolated and attached to fibronectin as described in Example 2. Seventy cells were micro-injected with an estimated 2-10 femtoliters of a 100 ng/microliter solution containing pGreen Lantern DNA in microinjection buffer (Experiment 93, Tables 2-3). Microinjection needles of 0.17-0.22 micron O.D. were employed. GFP expression was observed in 8 cells 5 hours post-microinjection. At 24 hours post-microinjection, 5 cells were positive for GFP expression.

#### EXAMPLE 5

##### COMPARISON OF INTEGRIN/FIBRONECTIN, CONCANAVALIN A AND ANTI-CD34 MEDIATED ATTACHMENT

The above methods of immobilization were compared as described below for their ability to strongly but reversibly immobilize non-adherent cells, especially CD34<sup>+</sup>, and to withstand the force of microinjection.

Four conditions were examined in this study:

(1) ICH-3 (biotinylated) anti-CD34 mAb [100 micrograms/ml] (very weak attachment, manual microinjection difficult);

(2) Con A [100 micrograms/ml] (strong attachment, manual microinjection possible, withstands force of automated microinjection);

(3) Pokeweed mitogen [100 micrograms/ml] (poor attachment);

(4) anti- $\beta 1$  integrin attachment to fibronectin (strong attachment, manual microinjection possible, withstands force of automated microinjection).

In two studies, attachment of CD34<sup>+</sup> cells to plates coated with biotinylated anti-CD34 monoclonal antibody was examined. In two studies, plates were first precoated with glutaraldehyde before addition of biotinylated anti-CD34 mAb (100 microgram/ml in PBS; ICH-3 antibody, CalTag). In one study, there was no precoating with glutaraldehyde. Cells attached minimally demonstrating a type of attachment described as "tethered". These cell types were relatively difficult to micro-inject.

Similar results were obtained for lectin *phytolecta americana* (pokeweed mitogen; 100 microgram/ml in PBS, Sigma) precoated with glutaraldehyde (as described below for Con A). CD34<sup>+</sup> cells were minimally attached, and could not be micro-injected.

There was strong attachment of CD34<sup>+</sup> cells to plates coated with Con A. Plates coated with Con A were prepared as follows:

1. Precoat dish with glutaraldehyde;
  2. Add 2.5% glutaraldehyde into cloning ring attached to tissue culture dish, let sit overnight at 4°C,
  3. Wash 4 times with sterile water,
  4. Add Con A, 9100 micrograms/ml in PBS (Sigma) to dish, at 37° C for 1 hour.
- Remove Con A.
5. Add cells in the colony ring, incubated 37° C for 30', then spun plate, or to fibronectin coated plates-in the presence of anti-beta 1 integrin activating antibody (TS2/16.2.1 hybridoma cell line; ATCC #HB-243; produces antibody reactive with integrin  $\beta_1$ -human CD29).

#### Biotinylated ICH-3

Results indicated a weak attachment of CD34<sup>+</sup> cells to the immobilization surface making manual microinjection difficult, but possible.

#### Con A

Results indicated a strong attachment of CD34<sup>+</sup> cells to the immobilization surface making both manual and automated microinjection feasible.

Pokeweed Mitogen

Results indicated weak attachment of CD34+ cells to the immobilization surface.

Anti-b, integrin antibody

- 5           Results indicated a strong attachment of CD34+ cells to the immobilization surface making both manual and automated microinjection feasible.

EXAMPLE 6IMMOBILIZATION OF U937 MYELOMONOCYTIC CELLS

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The ability of the described integrin/fibronectin immobilization method to attach a transformed leukemic cell line, specifically the human U937 myelomonocytic cell line is demonstrated in the present example. These cells normally grow continuously in culture as suspension cells. When U937 cells were simply added to fibronectin coated plates (fibronectin plates prepared according to our standard protocol) in the absence of antibody; 15 there was some attachment of cells to the fibronectin, and some manual microinjection was possible. When U937 cells were incubated with either the 8A2 or TS2/16.2.1 antibodies during culture with fibronectin coated plates, a significant improvement in the quality of attachment was noted. Again, manual microinjection was successfully demonstrated.

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EXAMPLE 7ATTACHMENT AND SPREADING OF SUSPENSION  
CULTURED CELLS TO INTACT HUMAN FIBRONECTIN IN THE PRESENCE  
OF TS2/16.2.1 MONOCLONAL ANTIBODY

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The kinetics of attachment and subsequent spreading of three suspension-grown cell types to intact human fibronectin (Fn) in the presence or absence of the activating anti-integrin monoclonal antibody (mAb) TS2/16.2.1 were investigated. The cell types studied thus far are U937, human myelomonocytic cell line; CEM, human lymphoblastic leukemia 30 cell line; and hSC, human primary hematopoietic CD34+ cord blood stem cells.

Commercially available culture dishes pre-coated with human Fn (Becton-Dickinson, product #40457) were used for this series of experiments. The Fn provided is in a denatured state due to the fact that the matrix is dried onto the dishes. U937 cells were plated into cloning rings placed on the dishes in the presence of the mAb at 37°C and allowed to attach



and spread for various times. The number of attached cells was determined by directly counting the number of cells in the cloning rings. In the presence of the mAb, U937 cells attached rapidly to Fn. Approximately 100% of the cells attached within 20 min. of mAb addition. Cellular projections, extensions, and micropseudopodia could be detected almost immediately (Fig. 13). The presence of cellular extensions correlated with firm attachment, not simple tethering, allowing for successful microinjection.

When CEM cells and hSCs (Fig. 15) were attached to the Fn coated dishes in the presence of the TS2/16.2.1 mAb, virtually 100% of the cells were attached within 30 and 90 minutes, respectively.

Thus far, these cell types did not readily attach to Fn in the absence of the activating mAb. This is the case regardless of the conformational state of the Fn. For example, as mentioned above, dishes pre-coated with dried denatured Fn have also been examined. Fn in an aggregated, though non-denatured, state was also prepared by coating dishes with Fn in PBS. A coating procedure which retains the native conformation of Fn was also employed in which the Fn is coated in a carbonate buffer.

#### EXAMPLE 8 ATTACHMENT AND SPREADING OF SUSPENSION GROWN CELLS TO COMMERCIALY AVAILABLE PEPTIDES BASED ON FN SEQUENCES.

The present example demonstrates the utility of the present invention for the use of peptides and mixtures of peptides ("cocktail") to promote the attachment of cells to a surface with reduced disruption to the cells. Rather than utilizing an entire adhesive molecule (e.g., fibronectin), portions of the molecule (produced by recombinant expression or protease digestion), a cocktail (mixture) of synthetic adhesive peptides that bind cell surface adhesion molecules may be used to coat the surface of a plate to which cells attached.

Various fragments of Fn were tested for their ability to attach suspension-grown cells. Retronectin (MW = 62,613; Rn) is a commercially available (Pan Vera, Corp; product #TAKT100A) recombinant human Fn fragment containing an RGD cell binding domain (type III repeat), a high affinity heparin-binding site, and the CS-1 site within the alternatively spliced IIICS region which contains the LDV amino acid sequence (Kimizuka, F. *et al.* (1991). The RGD and LDV amino acid sequenced are known to bind the  $\alpha_4\beta_1$  and

$\alpha_5\beta_1$  integrins, respectively. Rn has also been used to enhance the efficiency of retroviral gene transfer (Hananberg, H., *et al.*, 1996).

Culture dishes were coated with Rn in either PBS or carbonate buffer. U937 were plated onto the adhesive surfaces within cloning rings and the kinetics of attachment, spreading, and detachment (after mAb removal) were measured as described for the experiments on intact Fn. U937 cells bound to Rn in the presence of the TS2/16.2.1 mAb with more rapid attachment and spreading kinetics than to intact Fn. Cell attachment to and spreading on Rn occurred in the absence of activating mAb. The present study suggests that attachment and spreading on Rn was more efficient in the absence of the mAb. Similar results were obtained when hSC's were allowed to attach and spread on Rn (Fig. 17).

For attachment of cells expressing alpha 4/beta 1 or alpha 5/beta 1 integrins, dishes would also be coated with peptides (unmodified or perhaps chemically modified to increase the affinity for cell surface expressed adhesion molecules or to facilitate coating of culture dishes) containing amino acid sequences that are targets for attachment (e.g., sequences containing the LDV sequence of amino acids from the CS-1 region of fibronectin bind alpha 4/beta 1, and sequences containing the RGD amino acid sequence of fibronectin bind alpha 5/beta 1).

#### EXAMPLE 9 RETENTION OF HEMATOPOIETIC COLONY FORMING ACTIVITY BY INTEGRIN/FIBRONECTIN IMMOBILIZED CD34<sup>+</sup> CELLS

The present example demonstrates the utility of the invention for providing an effective mechanism for genetically modifying undifferentiated cell types, such as CD34<sup>+</sup> cells. In this manner, the present application also demonstrates the utility of the present invention for a method to provide gene therapy using cells that are provided to an animal, the cells being provided to the animal in an undifferentiated state and containing a particular gene or gene fragment thereof.

CD34<sup>+</sup> cells were immunomagnetically purified as described in Example 1 above. In a first study, the colony-forming ability of integrin/fibronectin immobilized CD34<sup>+</sup> cells (maintained overnight in an immobilized condition and subsequently released for colony assays) was compared with CD34<sup>+</sup> cells maintained overnight under similar culture conditions where the cells were not immobilized with integrin/fibronectin (Fig. 3). For

integrin/fibronectin immobilization, 2000 CD34<sup>+</sup> cells in IMDM/F-3-S were mixed with an equal volume of IMDM media conditioned 2 days by the TS2/16.2.1 hybridoma cell line, and plated into a well of a 96 well plate previously coated with fibronectin. The control well contained 2000 CD34<sup>+</sup> cells in IMDM/F-3-S alone. On the following day, cells (immobilized or control) were recovered from each well and plated in duplicate in 35 mm dishes containing 1 ml MethoCult GF culture media (containing 0.9% Methylcellulose, 30% fetal bovine serum, 1% bovine serum albumin, 10-4M 2-Mercaptoethanol, 2 mM L-glutamine, 50 ng/ml human SCF, 10 mg/ml human GM-CSF, 10, ng/ml human IL-3 and 3 units/ml Erythropoietin (StemCell Technologies Inc.); 16 days post plating, the number of CFU-derived colonies were assayed. For integrin/fibronectin immobilized cells, 70 BFU-E, 11 CFU-GM, 5 CFU-GEMM, 10 CFU-CFU-GM, 7 CFU-GEMM, 11 CFU-G, and 2 CFU-M per 1000 plated cells were likewise generated. The total number of CFU-GM, CFU-G, and CFU-M are totaled to give Amyeloid colonies, and both erythroid colony (BFUE-E) and myeloid colony results from immobilized cells are expressed as a percentage of the non-immobilized control (Fig. 3). In a second experiment, 3 wells of a 96 well plate were cultured overnight at 4° C, followed by 4 x washing with water, and then coated with 100 microgram/ml concanavalin A 2 hours at 37° C. Excess fibronectin and Con A were then removed. 2000 cells in 25 microliters IMDM/F-3-S were mixed with 25 microliters TS2/16.2.1 supernatant and added to each of the 3 fibronectin coated wells. 2000 cells in 50 microliters IMDM/F-3-S were added to each of 3 untreated wells (control). After overnight incubation, cells were recovered from each of two wells from each condition (i.e., integrin/fibronectin, Con A, control), and were plated in 1 ml methylcellulose-containing integrin/fibronectin immobilized cell: 183 BFU-E, 19 CFU-GM, 11 CFU-GEMM, 13 CFU-G, 24 CFU-M. For Con A immobilized cells: 155 BFU-E, 11 CFU-GM, 8 CFU-GEMM, 15 CFU-G, 17 CFU-M. For control cells: 169 BFU-E, 20 CFU-GM, 8 CFU-GEMM, 29 CFU-G, 27 CFU-M.

A third study was performed similarly to the second study. Non-immobilized cells were compared to integrin/fibronectin and Con A attached cells: integrin/fibronectin: 50 BFU-E, 35 CFU-G, GM, or M, 1.5 CFU-GEMM; Con A: 65 BFU-E, 26 CFU-G, GM, or M, CFU-GEMM; Control: 52 BFU-E, 50 CFU-G, GM, or M, 0.5 CFU-GEMM.

The results of the three integrin/fibronectin immobilization studies demonstrate no significant effect (either inhibitory or stimulatory) on the number or size of colonies derived from immobilized vs. control non-immobilized cells. Cells immobilized via Con A

demonstrated granulocyte/macrophage type colonies reduced approximately 50% with respect to control non-immobilized cells (Fig. 3).

EXAMPLE 10  
EFFECT OF EXPOSURE TO TS2/16.2.1 ON  
SELECTED CELLULAR FUNCTIONS

The effect of mAb exposure on three different cellular parameters (viability, proliferation, and retention of primitive stem cell activity) was investigated. U937 cells were seeded onto commercially available Fn-coated dishes in the presence or absence of the mAb. Viability was determined by trypan blue dye exclusion and cell proliferation was determined by direct cell counting with a hemocytometer. Treatment with the mAb did not affect cell viability. There was no significant difference in the viability of U937 cells in the presence (96.4%) or absence (95.8%) of the mAb. Similar results were obtained with CEM and hSC's (Fig. 16). Proliferation of U937 cells and CEM cells were unaffected by mAb exposure. The rate in the increase of cell number was similar in the presence or absence of mAb (Fig. 14). Preliminary studies indicate that hSC's can also proliferate in the presence of the mAb at the same rate as controls when stimulated with the appropriate cytokine cocktail (Fig. 16).

The effect of mAb exposure and subsequent attachment to and release from Fn and Rn on primitive hematopoietic stem cell activity in hSC's using the NOD/SCID reconstitution assay was also investigated. The present study indicated that attachment either to Fn after exposure to the activating mAb or to Rn without an activating mAb allowed for the retention of NOD/SCID reconstituting activity.

EXAMPLE 11  
USE OF VARIOUS REAGENTS FOR DETACHMENT OF CELLS FROM AN  
ADHESIVE SUBSTRATE.

Requirements for a cell detachment protocol:

1) is not toxic or damaging to cells; 2) does not trigger differentiation or other biological effects, causing the cells to lose stem cell activity; and 3) must be relatively rapid and efficient, releasing the majority of the cells in a short period of time (Fig. 2). This is particularly important where stem cells that are modified to include a nucleic acid sequence

of potential therapeutic action are to be transferred from a cell plate into an animal. By minimizing the damaging effects from physical and/or chemical disruption, the number of cells that remain viable is optimized, and hence the number of cells provided in the treatment is enriched.

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Spontaneous detachment of cells:

Since it is important to detach the cells with the least amount of cellular disruption, kinetics of cell detachment were investigated after simply removing the activating antibody. Detachment of the U937 cells from commercially prepared Fn-coated dishes (denatured Fn) occurred spontaneously after the mAb was removed by washing with buffer (Fig. 13). Approximately 50% of the cells detached within 24 hours of mAb removal and virtually 100% were detached by 48 hours. A more rapid rate of release could be achieved by gently applying a stream of buffer directly on the cells via a pipette. When U937 cells were plated on native or aggregated Fn the detachment occurred more slowly.

15 Detachment of either the U937 cell or hSC's did not readily occur on Rn after simply removing the mAb. This effect was seen whether Rn was coated in PBS or in the carbonate buffer.

hSC's were more resistant to spontaneous release after mAb removal. Only 40% of the cells spontaneously released from the Fn by 48 hours post-mAb removal (Fig. 15).  
20 However, the cells could be effectively released by pipetting, as described for the U937 above.

Detachment of cells using peptides based on fibronectin sequences:

It is anticipated that peptide sequences (unmodified or perhaps chemically modified to increase the affinity for cell surface expressed adhesion molecules) corresponding to targeted amino acid sequences in the adhesive (e.g., peptides containing the LDV or RGD sequences) may be employed as releasing agents to detach cells from an immobilized state. Again, it is anticipated that cell immobilization involving several adhesion/adhesive interactions may be best competed by a cocktail of peptides. By way of example, where  
30 retronectin is used to promote the attachment of cells, the following compositions alone or in combination may be used to detach the cells: 1. LDV sequences - provides interference

with  $\alpha_4 \beta_1$  binding to fibronectin; 2. RGD sequences - provides interference with  $\alpha_5 \beta_1$  binding to fibronectin.

Studies by the inventors have demonstrated that peptides containing LDV or RGD sequences promote the detachment of hSC's from both Rn and Fn.

5

#### Detachment of cells using antibodies:

Detachment of cells from an adhesive surface may also be accomplished by antibodies which interfere with the normal interaction between adhesion molecules and the adhesive (e.g., anti- $\beta_1$ , anti- $\alpha_4$ , anti-VLA-4, anti-CS-1 antibodies). Detachment may also  
10 be accomplished by mono- or poly-saccharides (or other charged molecules, e.g., lipids, polyanions and polycations) which may interfere with the normal interaction(s) between adhesion molecules and the adhesive.

#### Detachment of cells using various reagents:

15 Various reagents can be used to detach cells from an adhesive surface. For example,  
1. polyanions - may disrupt binding to the heparin binding site (eg. heparin, heparin sulfate, hyaluronan, other polysaccharides, etc.); 2. certain divalent cations (eg. calcium) - are known to disrupt integrin function; 3. certain chelating agents (eg. EDTA or EGTA) - are known to  
20 disrupt integrin function; 4) disintegrins - are naturally occurring soluble proteins, originally described from snake venom (Blobel, C.P., 1997), which contain integrin binding sequences;  
5. combinations of the above.

### EXAMPLE 12

#### METHOD OF PREPARING MICROINJECTION NEEDLES

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The present example outlines a method that may be used in the preparation of the microinjection needles of the present invention. As will be readily appreciated by one of ordinary skill in the art, this procedure may be employed to prepare a number of different diameter needles without the exercise of an undue amount of experimentation.

30

Fine glass microinjection needles of approximately 0.2 +/- 0.02 micron outer diameter were prepared from thin-walled borosilicate glass capillaries (Sutter, 1.2 mm O.D., 0.94 mm I.D.) with an automated pipette puller (Sutter, P-87, 3 mm box filament). Finer glass -

microinjection needles of average outer diameter 0.12 micron, 0.15 micron, or 0.17 micron were prepared from thick-walled borosilicate glass capillaries (Sutter, 1.2 mm O.D., 0.6 mm I.D.) with the Sutter P-87 pipette puller. Parameters for temperature, pull rate, and pressure were optimized for each needle size desired. In order to rapidly quality control the O.D. of  
5 needles immediately after pulling, we performed resistivity measurements on sample needles filled with electrolyte. By comparing the measured resistivity against a calibration curve, one can obtain a reasonable estimate of needle tip diameter. By SEM, verification was obtained that automatically pulled needles exhibit structural integrity and uniformity of tip geometry.

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### EXAMPLE 13 METHOD OF PREPARING A HOLDING PIPET

The holding pipette was prepared using a DeFonbrune microforge. Appropriate bends are made in a glass capillary so that it fits in a chuck assembly holder such that a 1-5 gram  
15 weight can be hung from the capillary positioned inside a heating filament. Heat is applied, softening the glass, resulting in the weight pulling the glass capillary to a 1-3 micron diameter, at which point the piece of glass capillary from which the weight is suspended breaks away leaving a holding pipette with a 1-3 micron diameter tip. The tip is then brought close to the heating filament, and the tip is heat polished resulting in a smooth tip with an  
20 opening of .5 to 2.5 microns. This holding pipette can then be attached to a syringe assembly that can be used to create a vacuum which will hold the cell in place for the microinjection procedure.

When making such a holding pipette, one is not limited to the DeFonbrune microforge. For example, any of the commercially available needle pullers (Sutter  
25 Instruments, Narishige, Kopf) can be used to pull a capillary to the above dimensions. However, the tip must still be heat polished and appropriate bends made in the holding pipette using a microforge (DeFonbrune or Narishige).

EXAMPLE 14  
MICROINJECTION METHOD EMPLOYING  
MICROINJECTION NEEDLE AND HOLDING PIPET

5        The present example details one method by which the present microinjection technique may be employed to introduce nucleic acid into the chromosomal DNA of a cell. In particular, the technique employs holding pipettes that stabilize cells through use of a vacuum. In some embodiments, the technique described here and variations thereof may be automated so as to provide a more rapid production of genetically modified cells.

10        As an alternative, an injection chamber that can have a vacuum behind a material with pores sufficient to hold numerous cells in place for subsequent injection may be employed. This chamber in some embodiments will be used in conjunction with an inverted microscope using phase contrast microscopy.

EXAMPLE 15  
Viability of U937 Cells After Microinjection Using  
Borsilicate or Quartz Needles

1.     Quartz Needles

**Table 4**

	Total Cells Injected	Cells Alive After 2h	Cells Alive After 24h	Cells Alive After 48h
25    Study One	57	35	30	27
	50	25	20	20
	50	32	29	25
30    Study Two	50	36	30	23
	62	48	41	36



	50	29	21	20
Total Survival	319	205	171	151
5 Percentage	100	64%	54%	47%

## 2. Borosilicate Needles

Table 5

5		Total Cells Injected	Cells Alive After 2h	Cells Alive After 24h	Cells Alive After 48h
	Study One	36	30	25	23
10		29	17	7	6
		68	42	14	10
	Study Two	56	36	27	20
15		30	19	18	18
		51	29	11	10
20	Study Three	52	26	20	20
		60	27	21	20
	Total Survival	382	226	143	127
25	Percentage	100	59%	37%	33%

**EXAMPLE 16**  
Viability of Stem CD34<sup>+</sup> After Microinjection  
Using Borosilicate or Quartz Needles

The present example demonstrates the utility of the present invention for providing enriched populations of viable, genetically modified cells, by microinjection.

## 1. Quartz Needles

Table 6

5		Total Cells Injected	Cells Alive After 2h	Cells Alive After 24h	Cells Alive After 48h
	Study One	50	12	9	7
10		50	16	10	Ring Moved
		50	18	15	15
		17	14	14	9
15	Study Two	46	32	11	7
		50	26	18	17
20		50	21	16	14
	Study Three	50	17	14	12
		50	22	16	12
25	Total Survival	263	178	123	93
	Percentage	100	68%	47%	44%

## 2. Borosilicate Needles

Table 7

	Total Cells Injected	Cells Alive After 2h	Cells Alive After 24h	Cells Alive After 48h
Study One	51	34	14	10
	54	32	16	10
Total Survival	105	66	30	20
Percentage	100	63%	29%	19%

Percent viability data obtained using quartz injection needles (Version 1.0Q- hSCs: 0.07 micron O.D.) and borosilicate injection needles (Version 1.0B-hSCs: 0.25 micron O.D.) injection needles. Such needles were used to inject U937 (Figs. 18 & 26) and primary human hematopoietic CD34<sup>+</sup> cord blood stem cells (Figs. 19 & 27). Such cells were prepared for injection as described in Example 1. The stem cells were injected intranuclearly with Oregon Green conjugated with Dextran (a detectable fluorescent molecule) and percent viability determined at 2, 24, and 48 hours. See Figures 18 and 19 for the viability studies and Figures 20 and 21 for the data regarding the outer diameter of the tips (as measured using a scanning electron microscope) of the needles used to perform the experiments. See Tables 4, 5, 6, & 7 above for the data used to produce the graphs shown in Figures 26 and 27. Figures 18 and 19 present data obtained in the first run study. Figures 26 and 27 present the cumulative data obtained in these and subsequent studies conducted under the same conditions.

The Version 1.0B and Version 1.0Q needles yield consistently higher viabilities than the data shown in Tables 2 and 3. It should be noted that these new data support and extend the data shown in Tables 2 and 3. All data shown in Tables 2 and 3 was collected while performing manual nuclear injections into CD34<sup>+</sup> cells, while the new data was collected while performing semi automatic nuclear injections into both CD34<sup>+</sup> and U937 cells.

It is important to note that for the most efficient gene therapy, it is important to demonstrate that the cells are attached sufficiently to use a semi-automatic or automatic microinjection system without disrupting the cells or significantly affecting cell viability, as this will allow for a large number of cells to be injected in a short time span. The data shown in Figures 18 and 19 support our previous studies and supports our claim that a semi-automatic or automatic injection system can be used to deliver DNA and proteins into the nucleus of CD34<sup>+</sup> cells as part of a gene therapy protocol.

An improvement in the Version 1.0B and Version 1.0Q needles as regards flow has been noted, when compared to the borosilicate needles used to generate the data shown in Tables 2 and 3 (early prototype needles made either on a DeFonbrune microforge or pulled using a different needle pulling program than has been developed for pulling the new needles). Although it was possible to inject cells using the early prototype needles, as seen by the data shown in Tables 2 and 3, routinely as many as 5 needles were required to perform approximately 50 injections. This prototype needle had flowing problems and plugged very easily. With the Version 1.0B and Version 1.0Q needles, the flow is improved and the Version 1.0B needle can be routinely used to inject greater than 50 cells/needle. The Version 1.0Q needle may be routinely used to inject greater than 25 cells/needle.

#### EXAMPLE 17

##### Preparation of Needles for Scanning Electron Microscopy:

Needle tips were sputter coated with gold-palladium in a SCD004 Bal-Tec sputter coater for 120 seconds at 15mA. The needle tips were then scanned and photographed using a Phillips 525M scanning electron microscope at 15KV. Outer diameter measurements were determined and the .046 micron coat value subtracted as the correction factor. Figure 28 represents photographs of scanning electron microscopic images of the 1.0B (28A) and 1.0Q (28B) needles.

**Table 8**  
**Scanning EM Data for Version 1.0 Q-hSC's Needle**

5		Column 1
	1.	.104
	2.	.129
10	3.	.154
	4.	.104
15	5.	.154
	6.	.054
	7.	.054
20	8.	.054
	9.	.030
25	10.	.030
	11.	.054
	12.	.054
30	13.	.030
	14.	.004
35	15.	.054

5	16.	.154
	17.	.129
	18.	.054
	19.	.054
	20.	.104
10	21.	.029
	22.	.054
	23.	.029
	24.	.029
	25.	.054
15	26.	.054
	27.	.079
	28.	.079
	29.	.054

30

The statistics generated from the above measurements appear below

35

Mean	Std. Dev.	Std. Error	Variance	Coef. Var.	Count
0.07	0.042	0.008	0.002	60.103	29
Minimum	Maximum	Range	Sum	Sum of Sqr	
0.004	0.154	0.15	2.019	0.19	

TABLE 9 (Scanning EM Data for Version 1.0B-hSC's Needle)

	<u>Needle</u>	<u>Diameter (Microns)</u>
	1	0.254
	2	0.304
5	3	0.304
	4	0.179
	5	0.179
	6	0.179
10	7	0.204

The statistics from the above measurements appear below:

Mean	Std. Dev.	Std. Error	Variance	Coef. Var.	Count
0.229	0.058	0.022	0.003	25.212	7
Minimum	Maximum	Range	Sum	Sum of Sqr	
0.179	0.304	0.125	1.603	0.387	

#### EXAMPLE 18 IMPROVEMENTS TO MICROINJECTION TECHNOLOGY.

##### Monitoring successful injections:

A. Resistivity measurements throughout the injection procedure accomplished by monitoring flow from the needle throughout the injection process modifying the needle holder as shown in Figure 25.

25 If the needle is flowing properly, we should observe a specific resistivity measurement based on the needle tip diameter, until the needle penetrates the cell at which time we expect to see (Brown, K.T., and D.G. Flaming: *Advanced Micropipette Techniques for Cell Physiology*, John Wiley and Sons, 1992, p. 157.).

30 B. For monitoring nuclear injections, include an expression vector that upon injection into the nucleus expresses a detectable protein that does not hurt the cell and can be detected in the living cell without perturbing cellular function (e.g., pCMV-GEP expression vector).

C. For monitoring either nuclear or cytoplasmic injections detectable molecules (e.g., Oregon Green conjugated with Dextran) can be injected and followed with time.

35 D. A sound device could be incorporated into the assembly that will produce an audible signal to indicate a successful injection (Figure 25).



Improvements of injection needles:

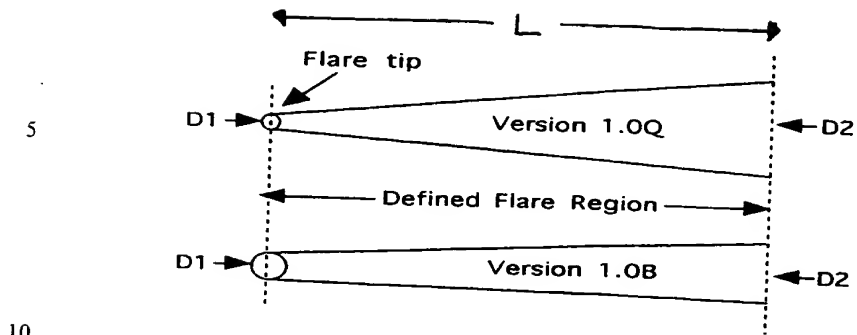
A. In some cases, the injection sample will be very sticky to the quartz or borosilicate and will immediately plug the needle, or coat the needle diluting out the molecule that you want to inject into the cell. We have worked out a procedure that allows us to siliconize these small needles including the Quartz Version with an average O.D. of .07 microns. We are following a procedure used to siliconize much larger injection needles used in generating transgenic animals (DePamphilis, M.L., Herman, S.A., Martinez-Salas, E., Chalifour, L.E., Wirak, D.O., Cupo, D.Y. and Miranda, M.: "Microinjecting DNA into Mouse Ova to Study DNA Replication and Gene Expression and to Produce Transgenic Animals" *BioTechniques*: 6: 662-680, 1988).

Injection pipettes are siliconized for 2-4 days in a desiccator with vapor from a small beaker of hexamethyldisilazane (Pierce). This procedure produces a monomolecular layer of silicon coating the total injection needle. Unfortunately, in the small needles that we are using, this procedure negates the capillary filling of the injection needle resulting in a large air bubble in the end of the needle that cannot be expelled. We have found that by making a small glass filament, we can suck the air bubble out, resulting in a flowing needle (Figure 22).

B. It is possible that using the Sutter Micropipette Beveler, a bevel may be applied to these needles again improving their % viability when used to inject the small stem cells.

C. In the case of some embodiments of small needles, Version 1.0Q and 1.0B (see Figure below), the flare of the needle is important as regards both loading of the needle and flow from the needle. The P2000 needle puller was used to pull the quartz needles. This resulted in both a smaller needle tip that had a greater flare.

Resistivity measurements from the Version 1.0B (O.D., 0.25 microns) needle have been obtained that equal those for the Version 1.0Q needle (O.D., 0.07 microns). The 1.0Q needle has a considerably smaller outer diameter. The Version 1.0Q needle has greater flare than that of the Version 1.0B and flows when appropriate pressure is applied, whereas a borosilicate needle having a similar outer diameter (0.07 microns) requires pressures that exceeds those that can be produced by this particular injection equipment.



Flare is an important characteristic that governs flow through the middle. As can be seen by looking at Table 10 and 12, respectively, the quartz injection needles (Version 1.0Q) have a greater flare than the borosilicate injection needles (Version 1.0B). The D1:D2 ratio for the borosilicate needles is in some embodiments an average of about 1:1.8 to about 1:3. The quartz needles (Version 1.0Q) has a D1:D2 ratio that is, in some embodiments, 1:3 to about 1:18. "L" as defined in the diagram is the distance (length) between the flare tip, D1, and the diameter, D2. In the actual needles measured for Table 2, the length between D1 and D2 was 1.3 microns.

20 Table 10 - Flare of Quartz Microinjection Needles, Version 1.0Q

	D1	D2	D1:D2 Ratio
1	0.104	0.784	1:6.800
2	0.129	0.704	1:5.500
3	0.154	0.754	1:4.900
4	0.104	0.704	1:6.800
5	0.154	0.654	1:4.200
6	0.054	0.404	1:7.500
7	0.054	0.454	1:8.400
8	0.054	0.254	1:4.700
9	0.030	0.454	1:15.100
10	0.030	0.254	1:8.500
11	0.054	0.254	1:4.700
12	0.054	0.604	1:11.200
13	0.030	0.204	1:6.800
14	0.004	0.254	1:63.500

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15	0.054	0.304	1:5.600
16	0.154	0.804	1:5.200
17	0.129	0.704	1:5.400
18	0.054	0.354	1:6.500
19	0.054	0.304	1:5.600
20	0.104	0.354	1:3.400
21	0.029	0.354	1:12.200
22	0.054	0.504	1:9.300
23	0.029	0.454	1:15.600
24	0.029	0.504	1:17.400
25	0.054	0.454	1:8.400
26	0.054	0.504	1:9.300
27	0.079	0.504	1:6.400
28	0.079	0.45	1:5.700
29	0.054	0.504	1:9.300

Table 11

X<sub>i</sub>: D2:D1 Ratio

20	Mean:	Std. Dev.:	Std. Error:	Variance:	Coef Var.:	Count:
	9.79	10.908	2.026	118.98	111.422	29
	Minimum:	Maximum:	Range:	Sum:	Sum of Sqr.:	# Missing:
	3.4	63.5	60.1	283.9	6110.73	14
25						

Table 12 - Flare of Borosilicate Microinjection Needles, Version 1.0B

30

35

40

	D1	D2	D1:D2 Ratio
1	.254	.604	1:2.300
2	.304	.554	1:1.000
3	.304	.654	1:2.150
4	.179	.504	1:2.800
5	.179	.504	1:2.800
6	.179	.554	1:3.100
7	.204	.504	1:2.400
8	.224	.604	1:2.700
9	.204	.404	1:2.000
10	.304	.554	1:1.800
11	.254	.554	1:2.200
12	.254	.654	1:2.600

5

10

13	.254	.554	1:2.200
14	.279	.604	1:2.200
15	.329	.704	1:2.100
16	.304	.554	1:1.000
17	.254	.504	1:2.000
18	.204	.504	1:2.500
19	.204	.554	1:2.700
20	.254	.554	1:2.200
21	.304	.604	1:2.000
22	.254	.554	1:2.200
23	.229	.504	1:2.200
24	.254	.254	1:2.400

20 A ratio of D1 to D2 in the range of about 1:about 1.5 to about 1:about 20, or about 1: about 2 to about 1: about 10 provide the D1:D2 width ratios according to some embodiments of the invention. In other aspects, the invention provides for widths on the average of about 1: about 7, or about 1: about 6, or about 1: about 5, or about 1: about 4, or about 1: about 3, or about 1: about 2.5, or finally, in the range of about 1:1.5 to about 1:2.5 are within the range of the present invention.

20

Table 13  
D2:D1 Ratio

	Mean:	Std. Dev.:	Std. Error	Variance:	Coef Var.:	Count:
25	2.298	.346	.071	.12	15.054	24
	Minimum:	Maximum:	Range:	Sum:	Sum of Sqr.:	# Missing
	1.8	3.1	1.3	55.15	129.482	0

30

D. The capillaries used in making the injection needles have a internal filament. This internal filament, among other things, helps the sample flow into the tip of the needle during the loading procedure. The loading process may also be aided by the filament increasing the capillary action. In loading the injection needles with small outer diameters, the needle should be loaded slowly. This will, among other things, introduce as few air bubbles into the tip of the needle as possible. If air bubbles are introduced, they

35

cannot be expelled from the needle and the needle must be replaced. The greater the D1:D2 ratio, the higher the number of needles that load effectively, having only minimal air bubbles introduced.

5 E. The hydration state of the small needles is also a factor to consider for proper loading of the needles. Optimally, the needle should be used the same day that it is pulled. In preparation for pulling the needle, the capillary may be attached to a vacuum and solutions drawn through the capillary. By way of example, the following fluids may be drawn through the capillary.

1. DriCote (Fisher) to make the glass hydrophobic,
- 10 2. Acetone
3. Filtered 70% Ethanol-dehydration

Other solutions well known to those of ordinary skill in the art may also be drawn up into the capillary and used in the preparation of the needles, given the present disclosure.

The capillaries are then baked at 200° C for up to an hour, and then pulled using  
15 either the P87 puller or the P2000 puller (both from Sutter instruments). The needles used the same day that they are pulled work most consistently as regards flow. With increasing time the needles decrease in usefulness. The needles do not flow after 2 days of storage. This may be due to particulate matter that was becoming attached through electrostatic interactions with time, thus clogging the needle. Although this is true in some cases, the majority of the  
20 needles are believed to lose their capacity to flow, at least in part because they become hydrated (especially in humid conditions). With time, when attempting to load the needle with injection sample, numerous air bubbles form in the tip of the needle. This will result in a needle that does not flow. With the present technology, one cannot achieve high enough pressures to expel the air bubbles. However, by simply baking the needle at 200° C for 1  
25 hour, one can restore the ability to load the needle with minimal air bubbles, thus restoring the flow.

#### EXAMPLE 19 CELL INJECTION WORK STATION

30

The present example defines the work station of the present invention for use in the microinjection of cells. While many different configurations of the work station will be

appreciated from the one described herein, the present description defines one particular configuration of the station as envisioned by the present inventors. The work station as provided in one embodiment is illustrated in FIG. 25. The work station comprises a microscope stage

5 One of the challenges in microinjection technology using the automatic injector is that the injector must define how far the needle penetrates the cell (set a Z value), without going through the cell, and contacting the hard tissue culture plastic. Sometimes, due to inconsistency in the surface of the tissue culture dish, the needle comes in contact with the tissue culture plastic, breaking the needle. There are several possible ways to get around this  
10 problem.

1. Place a pressure sensor in the micromanipulator that would indicate that the needle has contacted something harder than the cell.
2. Install a laser, sonar or radar device in the microinjection system that would monitor the needle tip position relative to the tissue culture dish, stopping the  
15 micromanipulator's motion whenever the tip comes close to the tissue culture dish surface.
3. Apply a transparent coating to the tissue culture dish that is of a composition that the needle could penetrate without breaking. We may actually be able to use the cell attachment molecules as part of this transparent coating.

20

#### EXAMPLE 20 MICROINJECTOR PLATES

Example A: Singhvi, R., A. Kumar, G.P. Lopez, G.N. Stephanopoulos, D.I.C. Wang, G.M. Whitesides and D.E. Ingber: "Engineering Cell Shape and Function" *Science* 264: 696-  
25 698 (1994).

The technique utilizes an elastomeric stamp (polydimethyl-siloxane) to imprint gold surfaces with pre-defined patterns (micrometer-sized) of self-assembled monolayers of alkanethiols. There may be a problem associated with this approach unless the stamp can be made transparent (See Example C; Fig. 23).

30 Example B: A manifold, containing 5-10 micron O.D. needles arranged in the same defined pattern as the manifold containing the injection needles, would be used to deposit microdroplets of solutions containing adhesion molecules onto to a culture surface (Fig. 24).

Cells could then be plated onto these adhesion islands, and the manifold containing the injection needles then used to inject the cells.

Example C: Mrksich, M., L.E. Dike, G.M. Whitesides: "Using Microcontact Printing to Pattern the Attachment of Mammalian Cells to Self Assembled Monolayers of Alkanethiolates on Transparent Films of Gold and Silver" *Exp. Cell Res.* 235:305 (1997). This technique utilizes the same principles as in Example A except that now transparent films of gold and silver are used, thus making it possible to use phase contrast microscopy to monitor the injections.

10

#### EXAMPLE 21 GENE THERAPY METHOD

The present example demonstrates the utility of the present invention for use in gene therapy protocols together with the herein described injection compositions and microinjection methodology. Employing same, an alternative method for the introduction of nucleic acid into the nucleic acid of a cell without the use of a carrier, such as a retrovirus, adenovirus, or other carrier cell will be provided.

Gene therapeutic applications of stem cell microinjection to include the following elements: Approximately  $1-10 \times 10^3$  highly enriched stem cells will be obtained from blood, and will be temporarily immobilized. Microinjection of these cells will deliver a reproducible volume-containing DNA and possibly integration enzyme(s) - such that 1-3 copies of the DNA are successfully integrated per cell. Microinjected DNAs of 15-25 kb in size, containing two independently regulated transgenes, will be integrated without rearrangement. One transgene, targeted for expression in stem cells, will provide for *in vitro* (e.g., rsGFP, or truncated nerve growth factor receptor; tNG-R) or *in vivo* (e.g., MGMT) selection of transduced stem cells. The therapeutic transgene (e.g., DNA for ADA SCID, globin for hemoglobinopathies, MDR-I for chemoresistance) will be targeted for expression in the appropriate hematopoietic cells.

30

The above is a detailed description of particular embodiments of the invention. Those of skill in the art should, in light of the present disclosure, appreciate that obvious

modifications of the embodiments disclosed herein can be made without departing from the spirit and scope of the invention. All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. The full scope of the invention is set out in the claims that follow and their  
5 equivalents. Accordingly, the claims and specification should not be construed to unduly narrow the full scope of protection to which the present invention is entitled.



SEQUENCE LISTING

SEQ. ID NO. 1:

H-Glu-Ile-Leu-Asp-Val-Pro-Ser-Thr-OH

5

SEQ. ID NO. 2:

H-Arg-Gly-Asp-Ser-OH

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CLAIMS

What is claimed is:

- 5 1. A non-viral mediated method for incorporating a macromolecule into a cell comprising:  
immobilizing stem cells or progenitor cells onto a surface that includes an  
adhesive protein fragment capable of supporting attachment of cells to a  
surface to provide immobilized stem cells or progenitor cells; and  
10 introducing a molecule into the immobilized cells to provide an enriched  
population of modified stem cells or progenitor cells.
2. The method of claim 1 wherein the cells comprise hematopoietic stem cells.
- 15 3. The method of claim 1 wherein the surface is treated with an adherent molecule selected from the group consisting of fibronectin, collagen, laminin, epiligrin, invasin, osteospondin, thrombospondin, proteoglycan, glycosaminoglycan, UCAM, ICAM and VCAM-1
- 20 4. The method of claim 3 wherein the adhesive molecule fragment is a fragment of fibronectin.
5. The method of claim 4 wherein the cell-adherent promoting fragment of fibronectin is a retronectin molecule.
- 25 6. The method of claim 1 wherein the nucleic acid is introduced into the cell by microinjection.
7. The method of claim 1 wherein the molecule is a nucleic acid molecule and  
30 wherein the enriched population of cells are further defined as genetically modified cells.



8. The method of claim 1 wherein the nucleic acid comprises a sequence encoding a therapeutic gene.
9. The method of claim 1 wherein the macromolecule is introduced into the cell by  
5 microinjection with a microinjector needle having a flare region, said flare region having a flare tip having a diameter D1 and a diameter D2, wherein the ratio of D1:D2 is about 1:2 to about 1:20, and wherein the length between D1 and D2 is L.
10. A method for incorporating a molecule into a population of cells comprising:  
10 treating a surface suitable for attachment of cells with a preparation comprising a cell-adhesive molecule or fragment thereof to provide a treated surface;  
exposing said treated surface to a population of non-adherent cells for a time sufficient to promote attachment of the cells capable of resisting detachment upon microinjection; and  
15 introducing the molecule into the attached cells to provide a population of modified cells.
11. The method of claim 10 wherein the cells comprise hematopoietic stem cells.
- 20 12. The method of claim 10 wherein said population of non-adherent cells are treated to provide treated cells having expressed integrins capable of adhesion of the treated cells to the surface.
13. The method of claim 12 wherein the treated surface is treated with an adhesive  
25 molecule of fibronectin or a cell-adhesive fragment thereof.
14. The method of claim 13 wherein the cell-adhesive fragment of fibronectin is retronectin.
- 30 15. The method of claim 10 wherein the non-adherent cells are hematopoietic stem cells that include a therapeutic gene.

16. An apparatus for injecting cells comprising:  
a microinjection needle having a flare region and a flare tip, wherein said flare tip  
has an outer diameter of about 0.05 microns to about 0.5 microns; and  
a plate capable of supporting the attachment of cells, said plate having thereon a  
fixed pattern of a bio-adhesive molecules.
17. The apparatus of claim 15 wherein the fixed pattern includes plate areas without  
bioadhesive molecules.
18. The apparatus of claim 16 further comprising a multi-microneedle manifold  
capable of securely holding multiple microinjection needles.
19. A kit comprising:  
a carrier means adapted to contain at least two container means;  
a first container means comprising a microinjection needle having a flare region  
comprising a flare tip, said flare tip having an outer diameter of about 0.05  
to about 0.50 microns; and  
a second container means comprising a cell-adhesive molecule or a fragment  
thereof.
20. The kit of claim 19 wherein the flare tip of said microinjection needle is further  
defined as having an outer diameter of about 0.05 microns to about 0.15 microns.
21. The kit of claim 19 further comprising a plate suitable for the attachment of cells,  
wherein said plate includes a grid pattern having dimensions suitable for containing an  
individual cell therein.
22. The kit of claim 21 wherein the plate is further defined as comprising a series of  
wells, wherein said wells include an adhesive molecule capable of immobilizing a cell  
onto said wells.

23. The kit of claim 19 further comprising a third container means comprising a detachment reagent.

24. The kit of claim 21 wherein the adhesive molecule fragment is a fragment of a molecule selected from the group consisting of: fibronectin, collagen, laminin, epiligrin, invasin, osteospondin, thrombospondin, proteoglycan, glycosaminoglycan, UCAM, ICAM and VCAM-I.

25. A multiple microinjection apparatus comprising:  
a microinjection needle holding head; and  
a plurality of microinjection needles having a flare region and comprising a flare tip, wherein said flare tip has a bore diameter of about 0.05 to about 0.5 microns,  
wherein said plurality of microinjection needles are spaced equidistant from one another and in a grid orientation on said microinjection needle holding head.

26. The multiple microinjection apparatus of claim 25 wherein the tip of said microinjection needle is further defined as having an outer bore diameter of about 0.05 microns to about 0.2 microns.

27. The multiple microinjection apparatus of claim 25 further comprising a plurality of holding pipettes, said holding pipettes having a tip with an inner bore diameter of about 0.5 to about 2.5 microns.

28. The multiple microinjection apparatus of claim 25 wherein said apparatus comprises 96 microinjection needles.

29. The multiple microinjection apparatus of claim 25 further comprising a cell attachment surface, said surface comprising a substrate suitable for the attachment of cells, said substrate including a grid configuration thereon.

30. The multiple microinjection apparatus of claim 29 wherein the cell attachment surface comprises a multiplicity of wells.
31. The multiple microinjection apparatus of claim 30 wherein the multiplicity of wells contain an adhesive molecule.
32. The multiple microinjection apparatus of claim 31 wherein the adhesive molecule is fibronectin.
33. A needle having a flare region comprising a flare tip D1 and an endpoint of said flare region, D2, wherein the ratio of D1:D2 is about 1:1.5 to about 1:20.
34. The needle of claim 33 wherein the ratio of D1:D2 is about 1:2 to about 1:10.
35. The needle of claim 33 wherein the ratio of D1:D2 is about 1:2 to about 1:5.
36. The needle of claim 33 further defined as a quartz needle or a borosilicate glass needle.
37. A method for detaching an attached cell population at a substrate surface without substantial loss of cell viability comprising:  
    exposing a population of attached cells on an adhesive to a preparation comprising a peptide capable of competing with the attached cell for an attachment site on said surface, for a period of time sufficient to permit detachment of said cells; and  
    detaching said attached cells from the substrate,  
wherein the cell viability of detached cells is about 50% to about 95% of the attached cell population.
38. The method of claim 37 wherein the preparation comprises a peptide or mixture of peptides, said peptides having a sequence RGD, LDV, or a combination thereof.

39. The method of claim 38 wherein the population of attached cells are hematopoietic cells.

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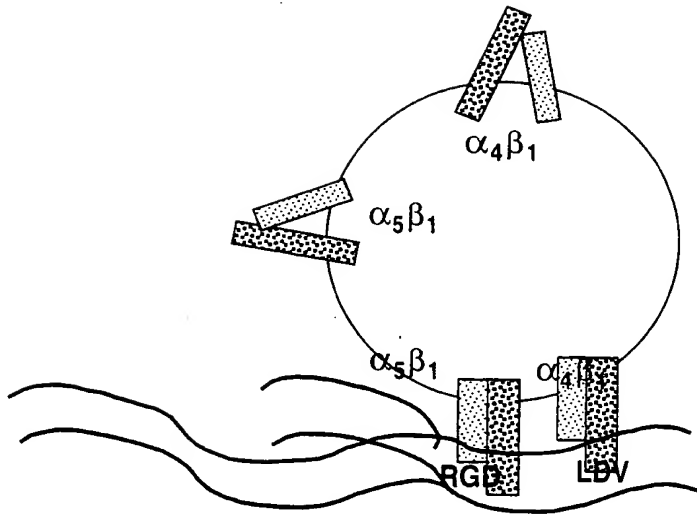


Fig. 1A

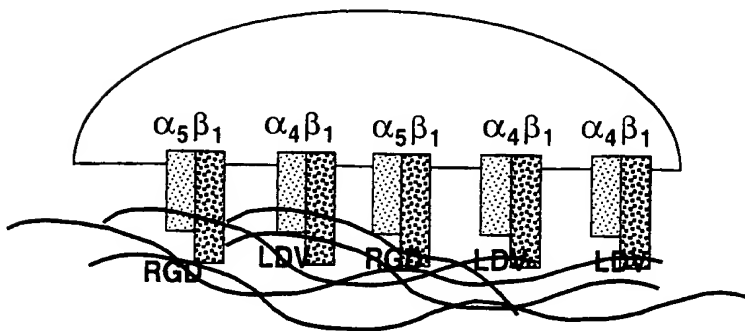


Fig. 1B

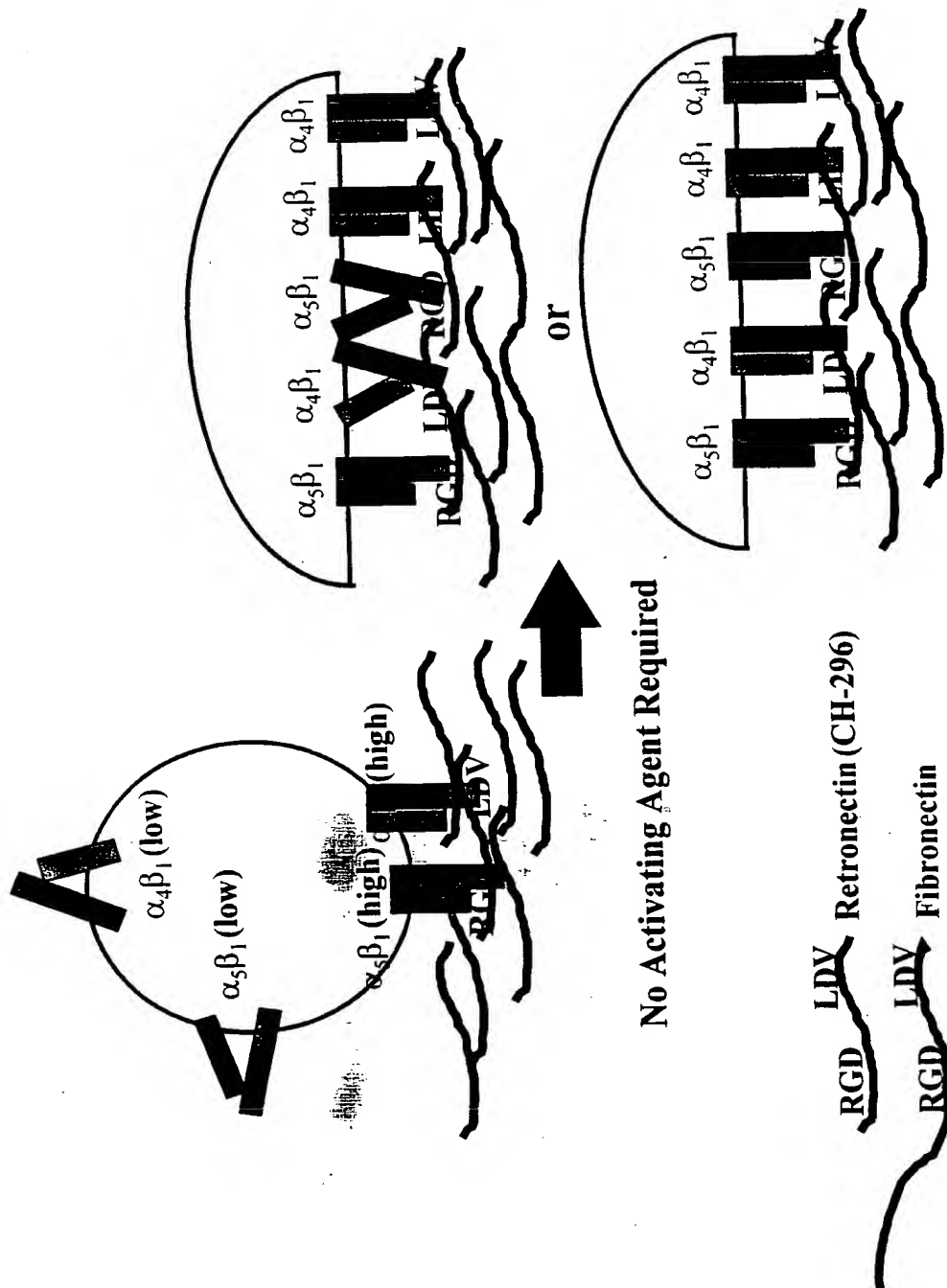


Fig. 1C

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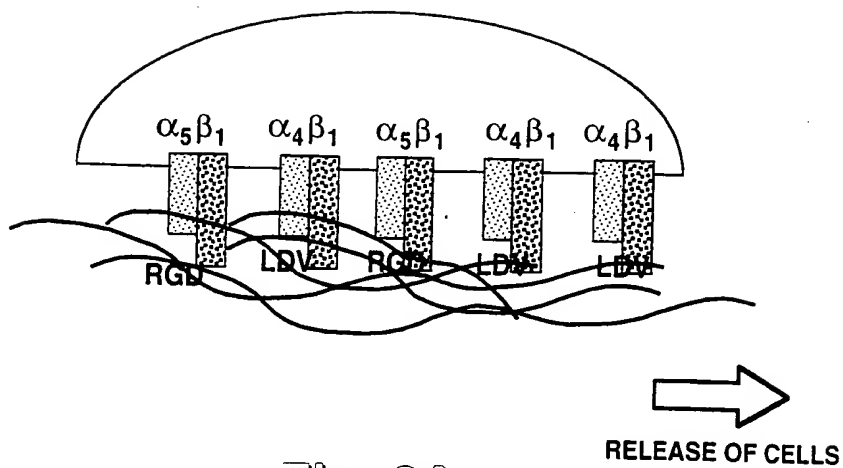


Fig. 2A

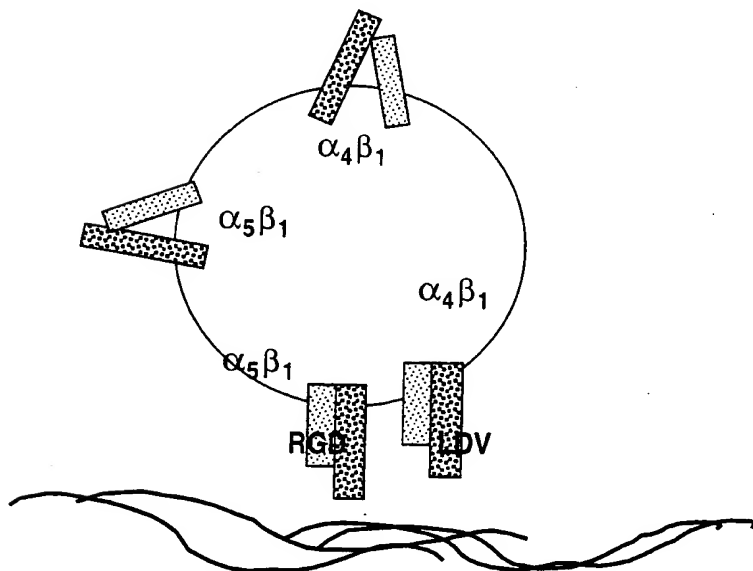


Fig. 2B



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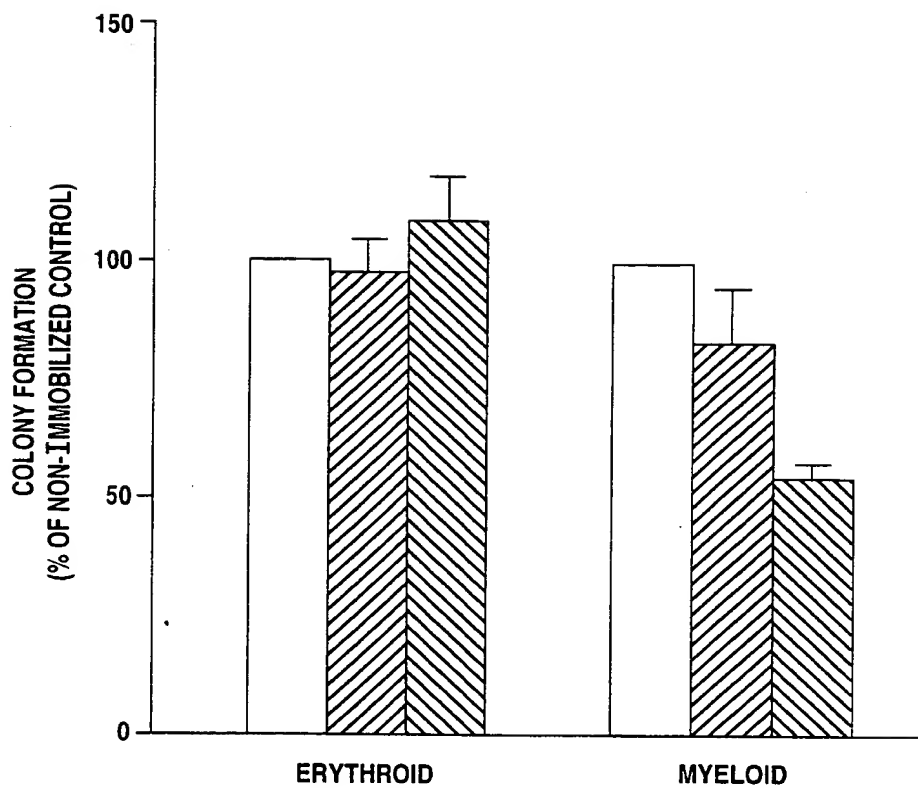


Fig. 3

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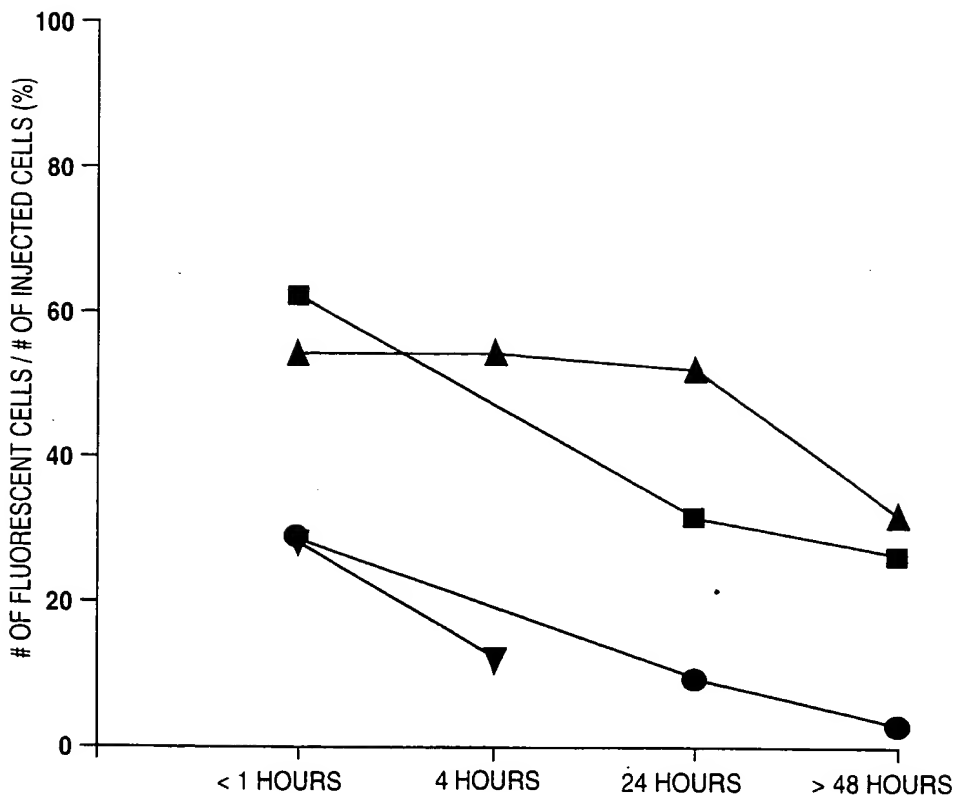


Fig. 4

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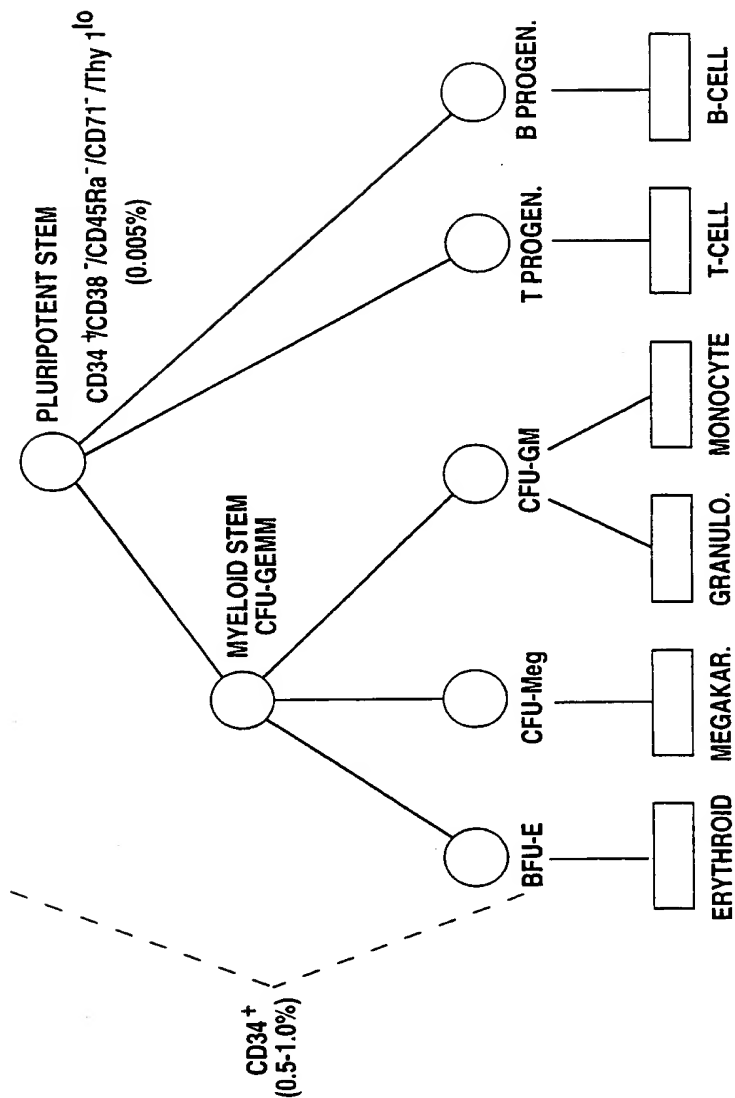


Fig. 5

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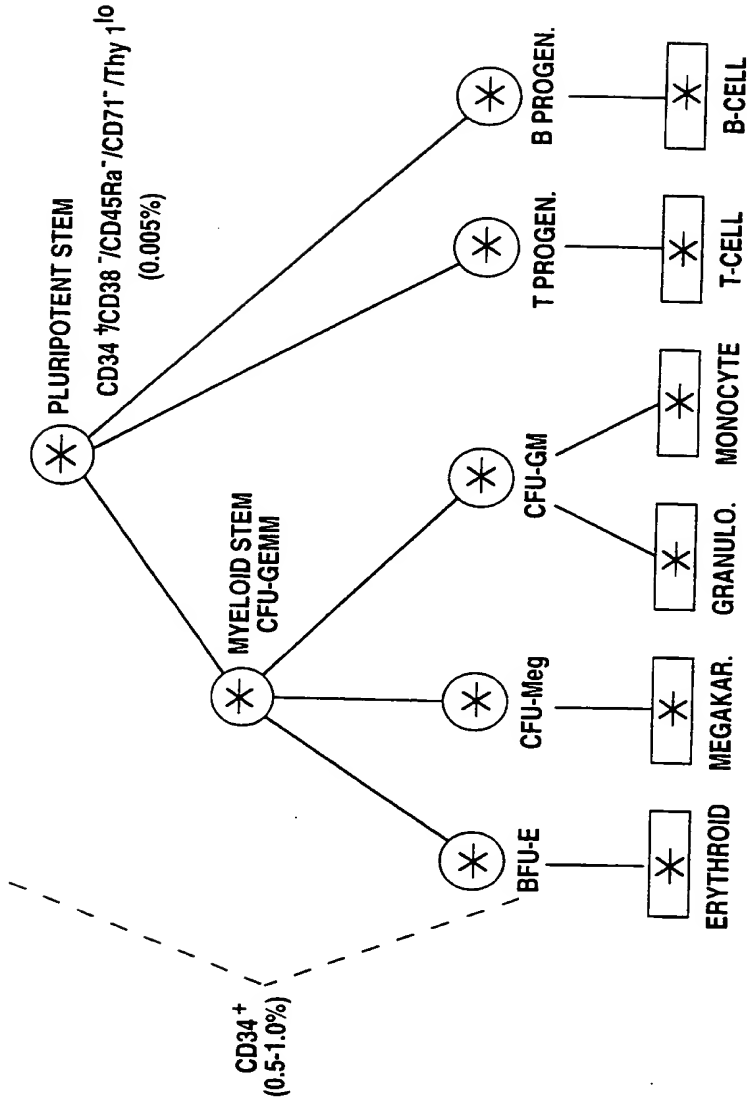


Fig. 6

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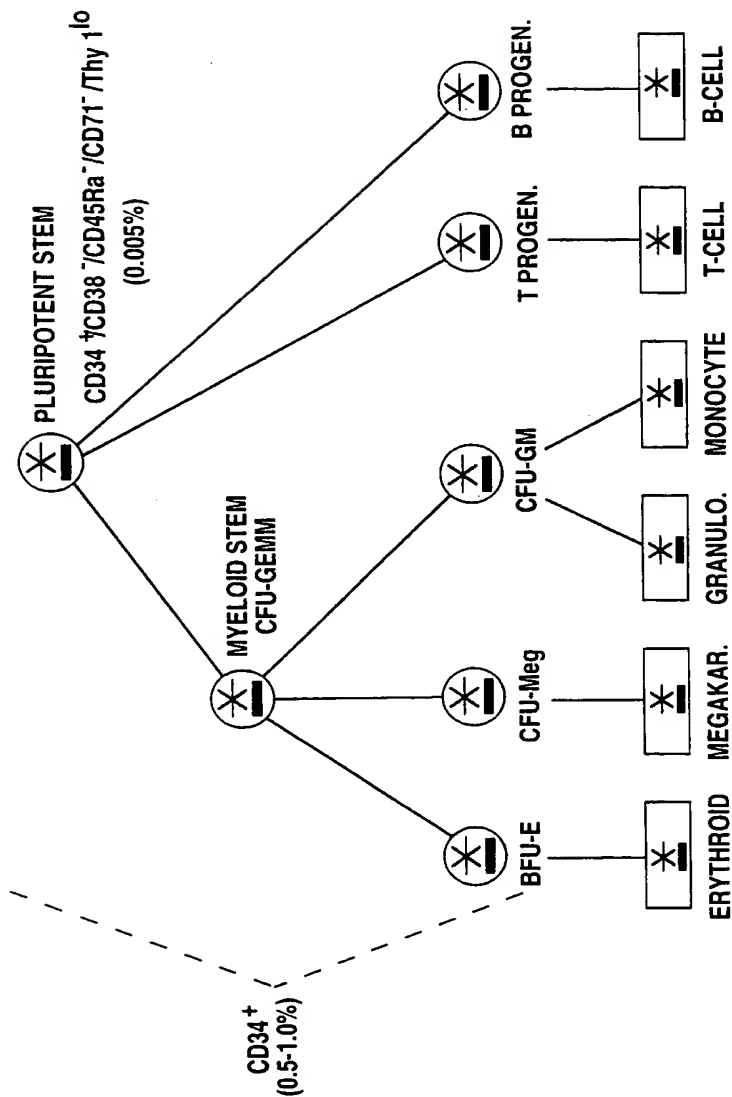


Fig. 7

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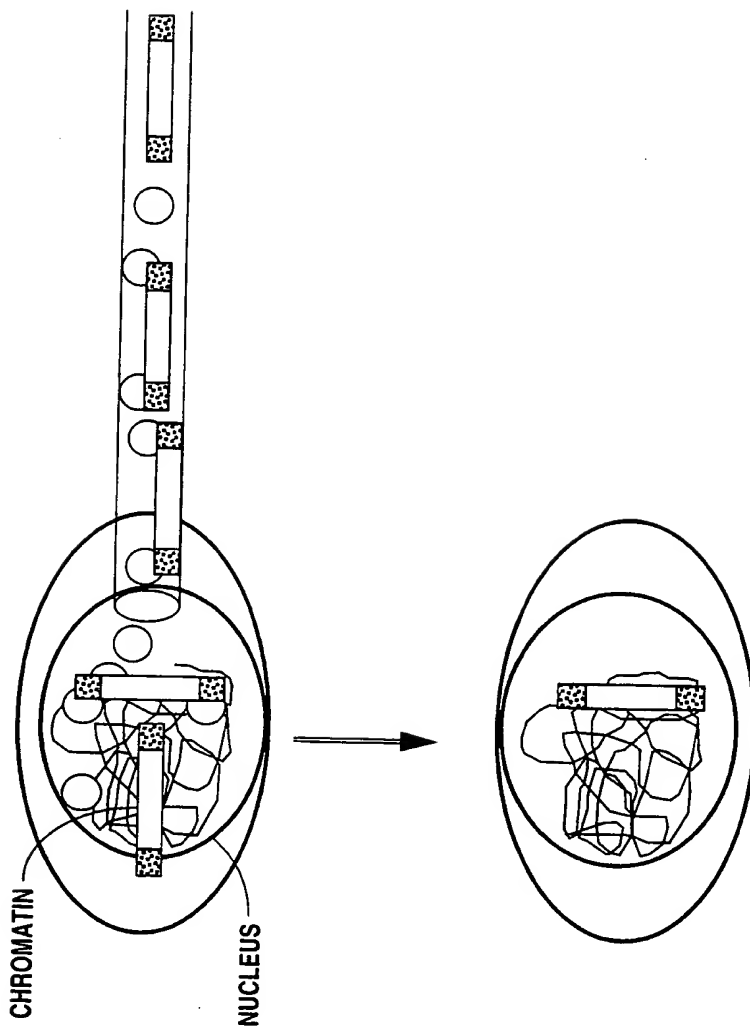


Fig. 8

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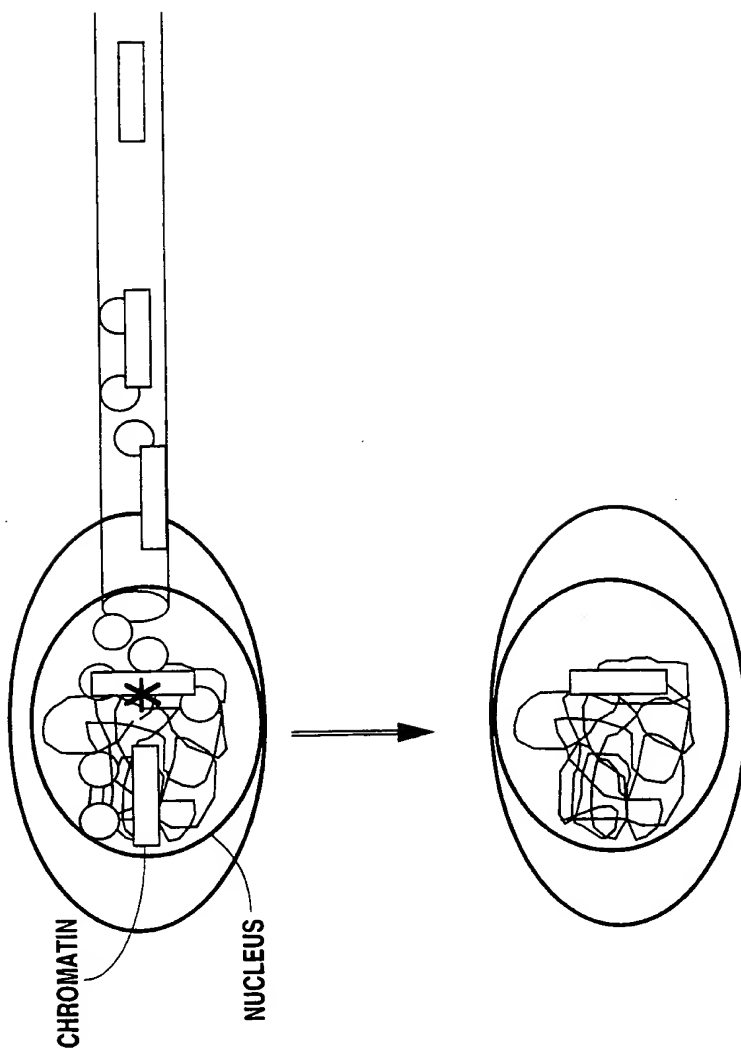


Fig. 9

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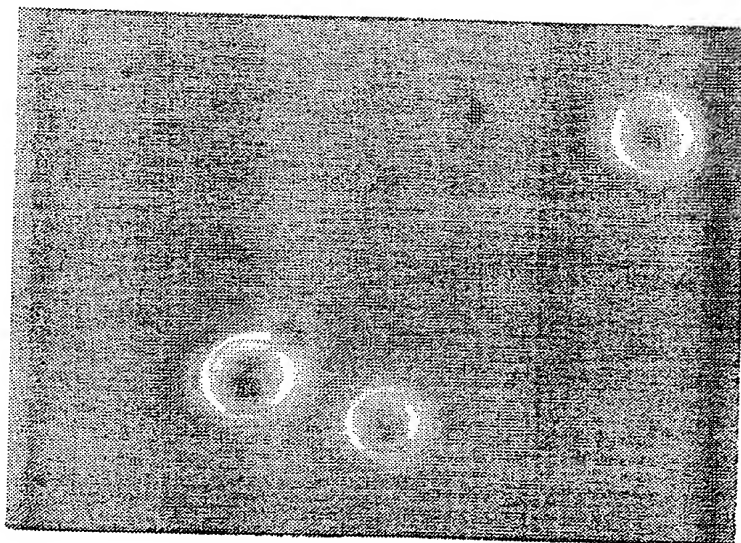


Fig. 10A

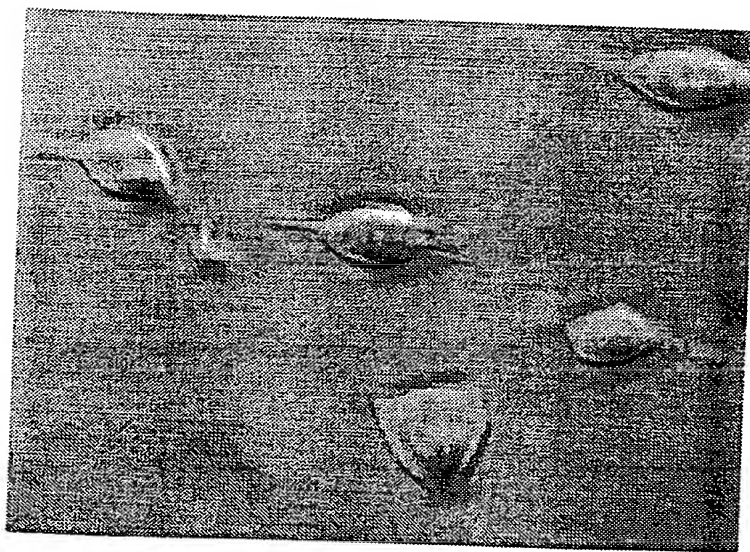


Fig. 10B



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Fig. 11

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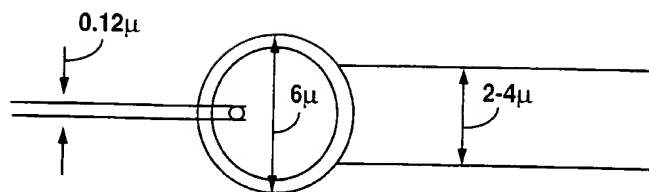


Fig. 12A

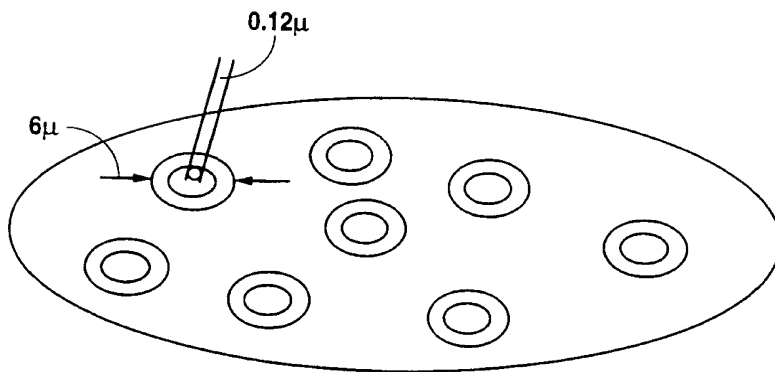


Fig. 12B

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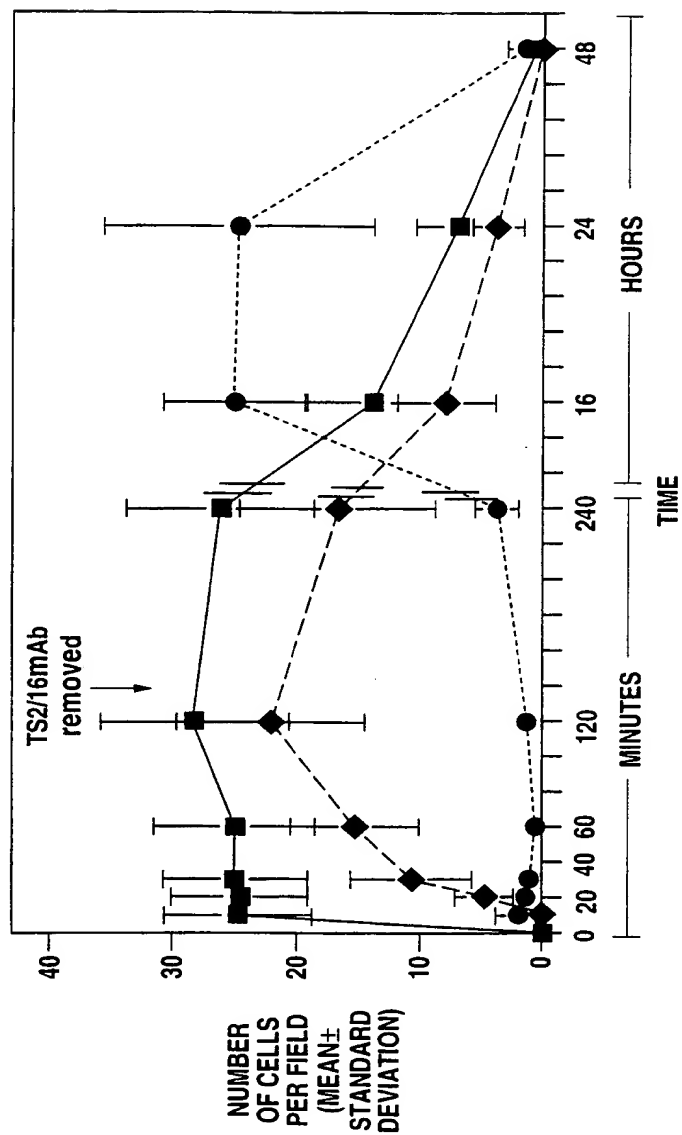


Fig. 13

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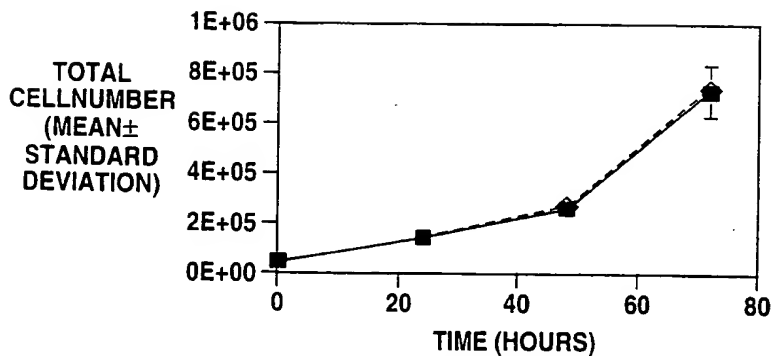


Fig. 14

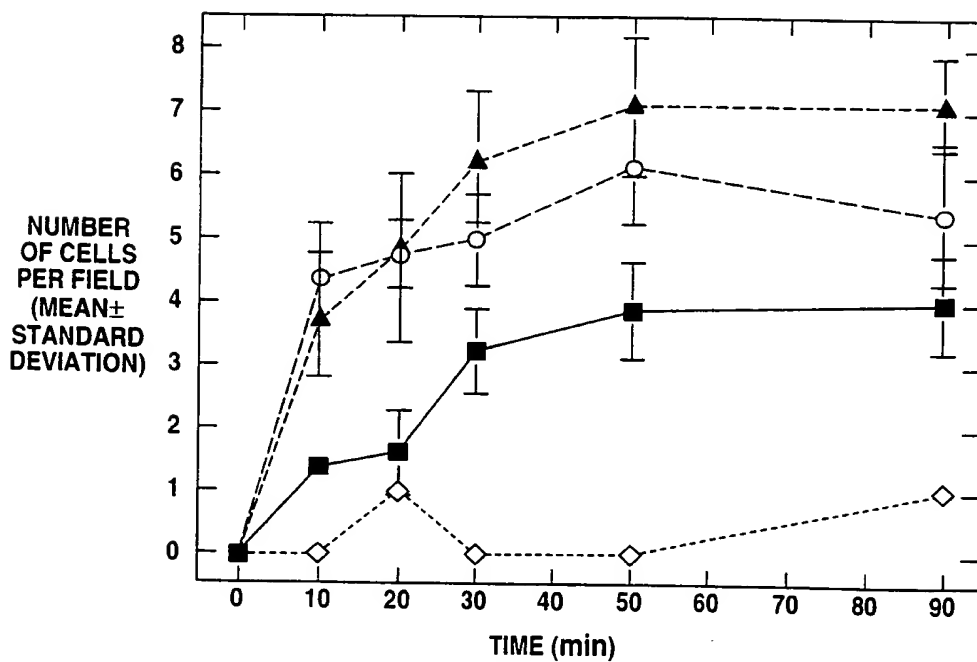


Fig. 17

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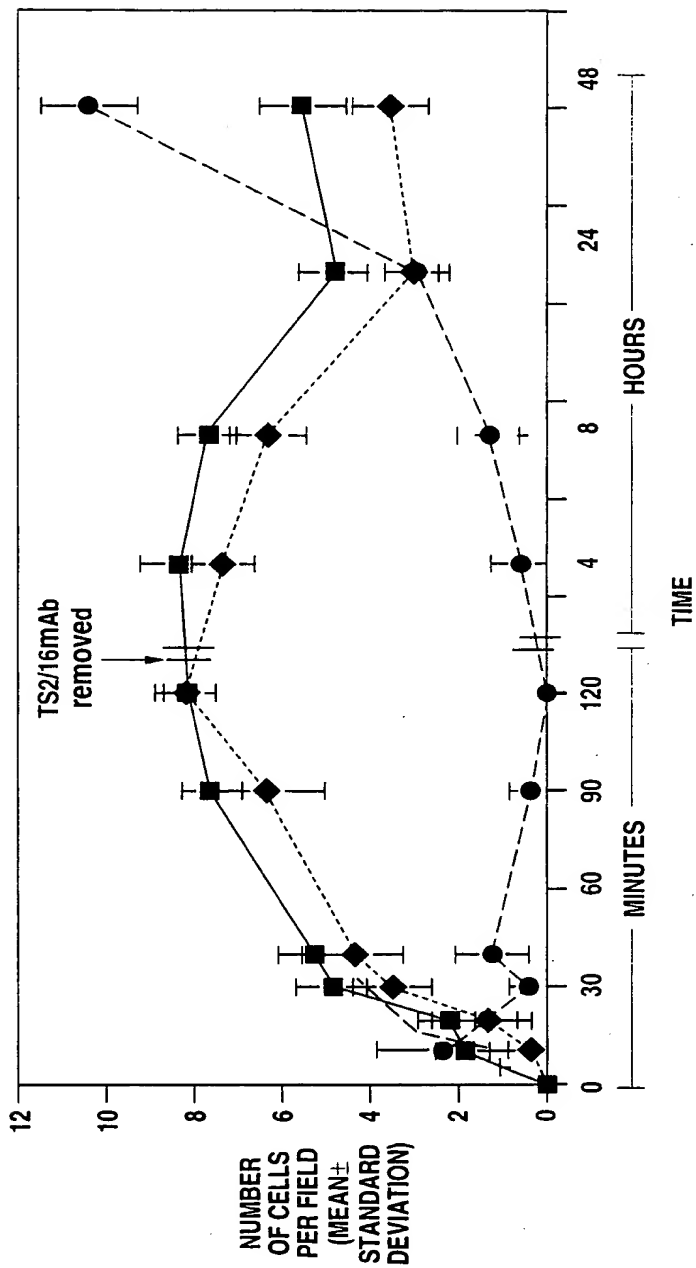


Fig. 15

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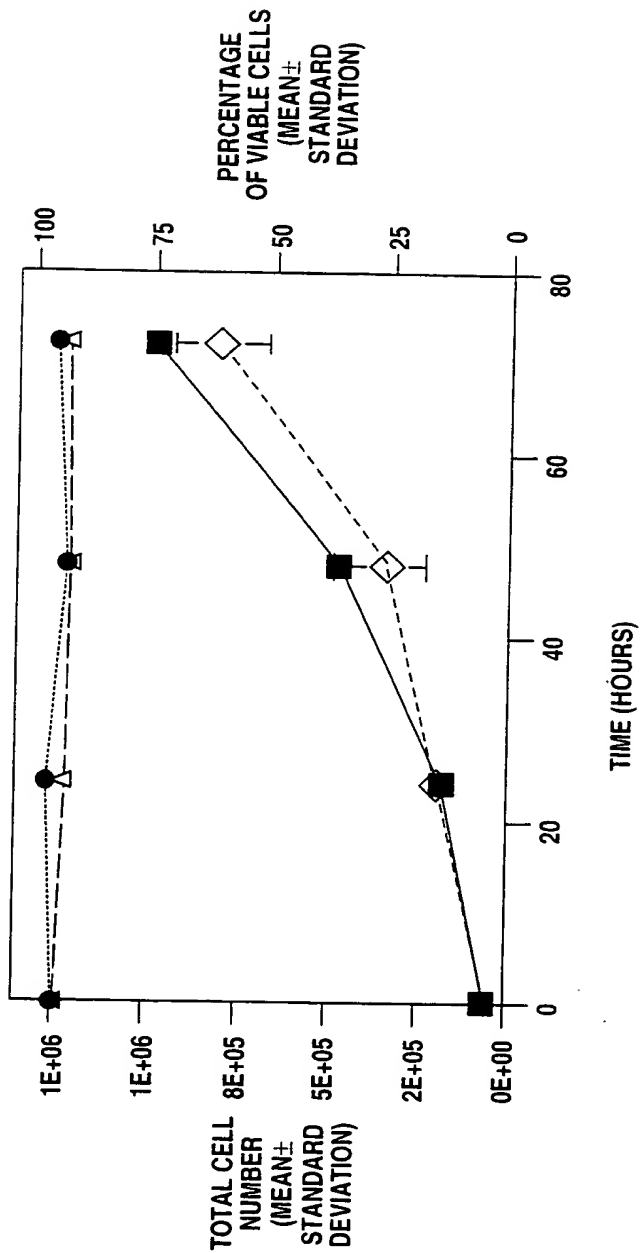


Fig. 16

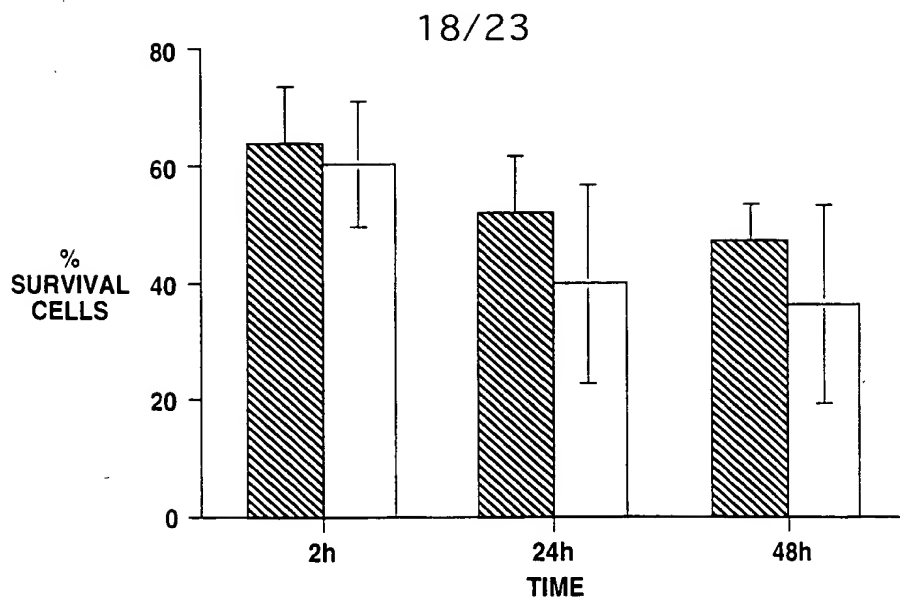


Fig. 18

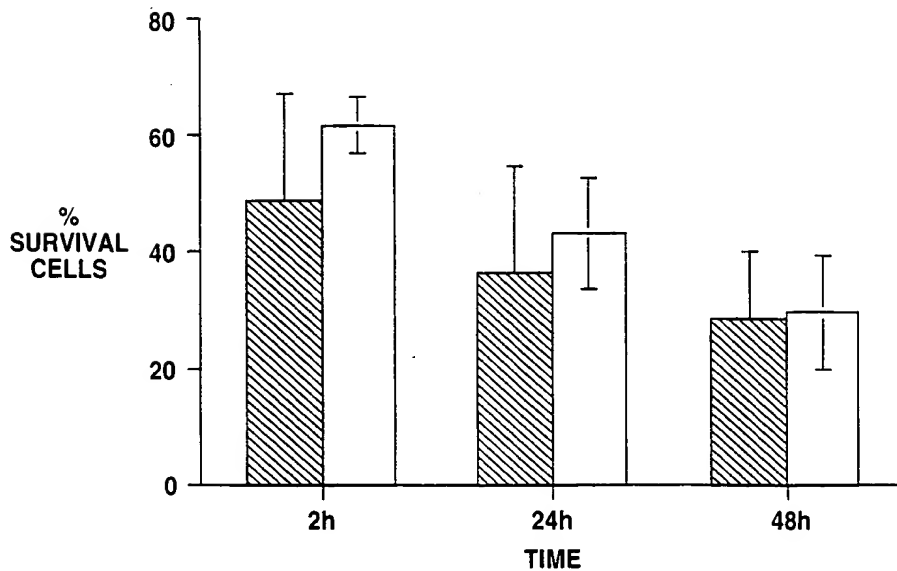


Fig. 19

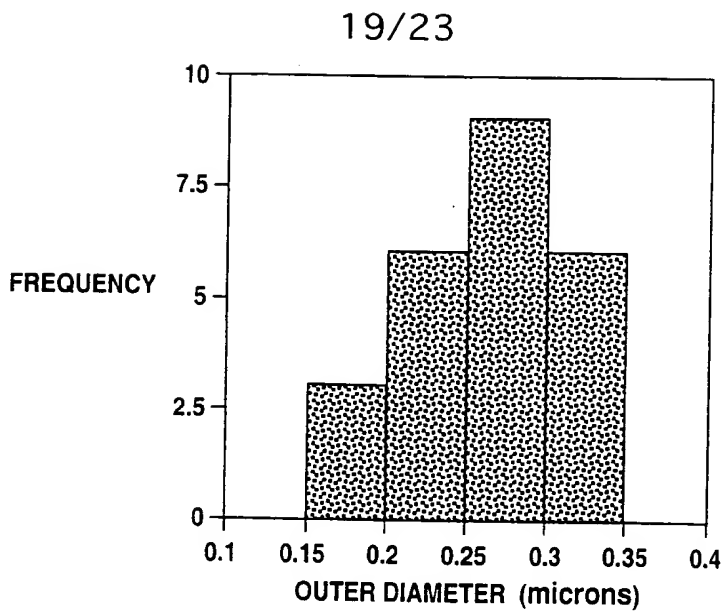


Fig. 20

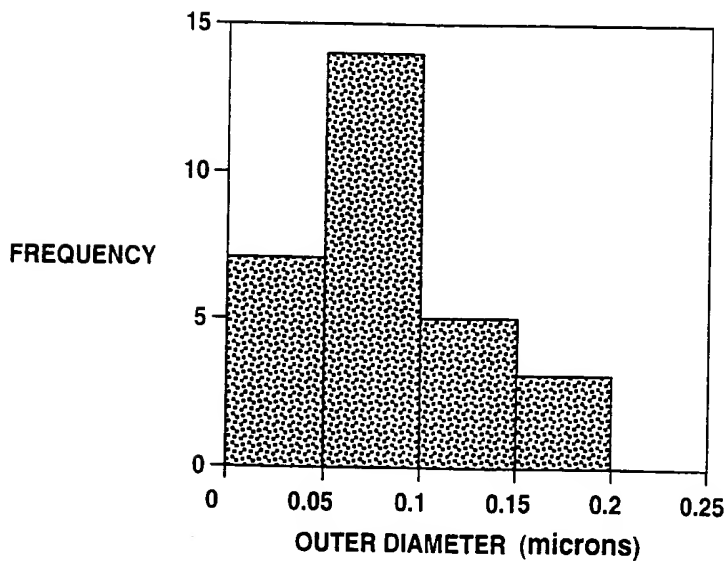


Fig. 21



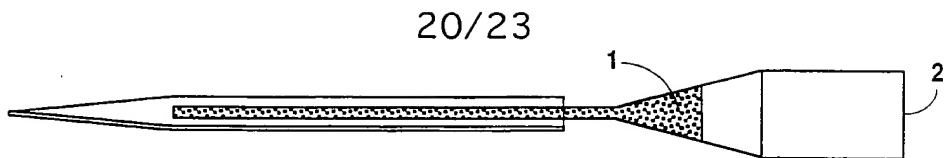


Fig. 22A



Fig. 22B

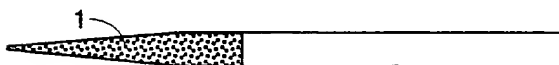


Fig. 22B'

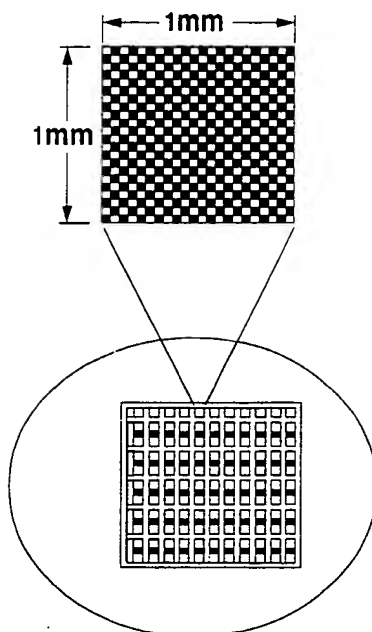


Fig. 23

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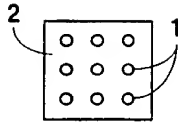


Fig. 24A

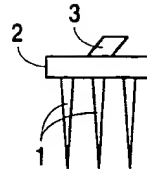


Fig. 24B

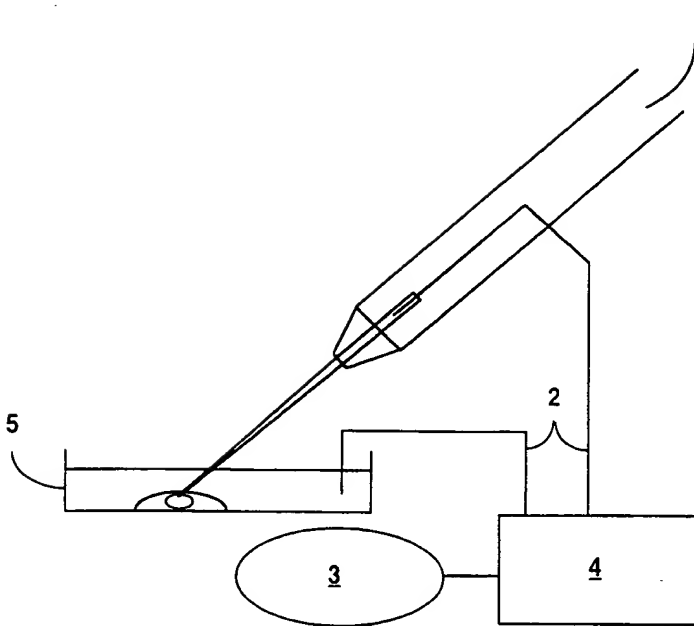


Fig. 25

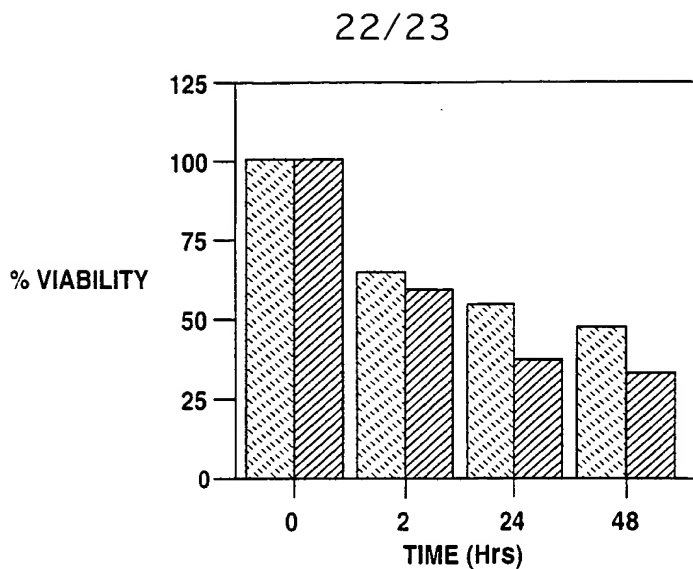


Fig. 26

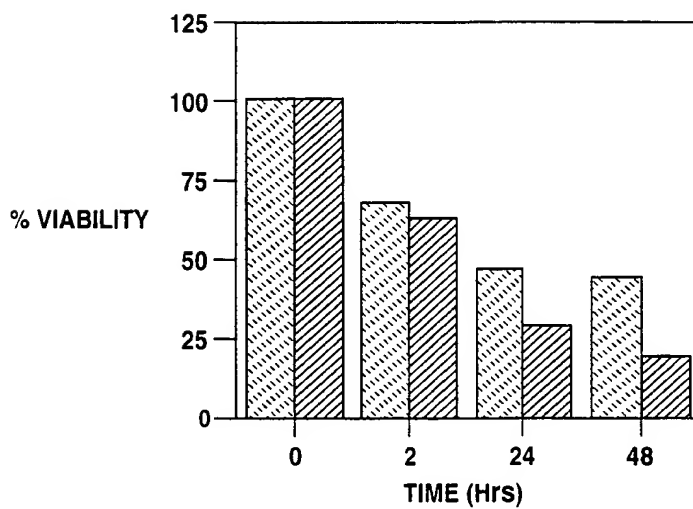


Fig. 27

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Fig. 28A

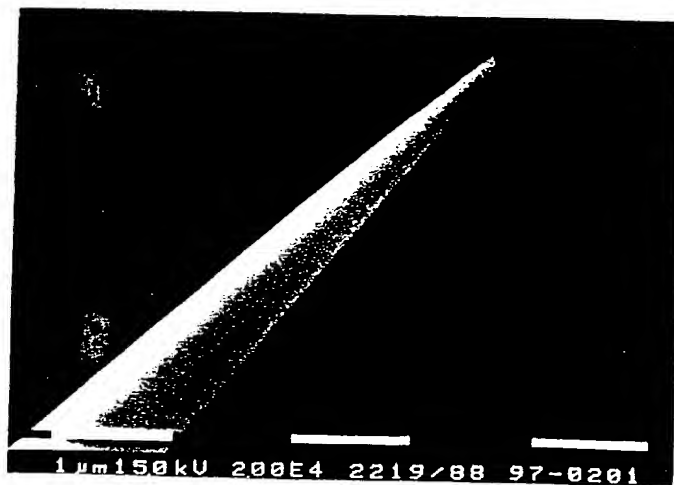


Fig. 28B

## INTERNATIONAL SEARCH REPORT

National Application No.

PCT/US 97/23781

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12M3/00 C12N15/89

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12M C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 463 508 A (GRACE W R & CO) 2 January 1992 cited in the application	1,3-8,10
Y	see column 1, line 1 - column 3, line 11	2,11,24, 32
A	see column 5, line 6 - column 6, line 50	16-18
Y	--- WILLIAMS D A ET AL: "FIBRONECTIN AND VLA-4 IN HAEMATOPOIETIC STEM CELL -MICROENVIRONMENT INTERACTIONS" NATURE, vol. 352, 1 August 1991, LONDON GB. pages 438-441, XP002050847 see the whole document ---	2,11,24, 32

-/--

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.**\* Special categories of cited documents:**

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search

20 April 1998

Date of mailing of the international search report

15/05/1998

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De Kok, A

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 97/23781

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WAYNER E A ET AL: "Activation-dependent recognition by hematopoietic cells of the LDV sequence in the V region of fibronectin" THE JOURNAL OF CELL BIOLOGY., vol. 116, no. 2, January 1992, ROCKEFELER UNIVERSITY PRESS US, pages 489-497, XP002062644 cited in the application	37-39
A	see the whole document	1-4, 10-13, 24, 32
A	WO 96 17073 A (TAKARA SHUZO CO) 6 June 1996 see abstract	2, 11, 24, 32
P.A	-& EP 0 795 606 A (TAKARA SHUZO CO.LTD.) 17 September 1997 see page 2, line 31 - page 5, line 10	2, 11, 24, 32
A	EP 0 292 899 A (ZEISS CARL FA ) 30 November 1988 see the whole document	16-18, 25-32
A	EP 0 175 966 A (CALGENE INC) 2 April 1986 see page 6, line 13 - page 8, line 17	25-32
A	US 5 457 041 A (GINAVEN ROBERT O ET AL) 10 October 1995 see column 3, line 26 - column 5, line 16 see column 7, line 5 - line 38; figure 6	19-32
A	WO 91 05519 A (US ARMY) 2 May 1991 see the whole document	19-32

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/23781

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